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STUDIES ON THE METACERCARIA OF FASCIOLA HEPATICA L.:

ENCYSTMENT AND EXCYSTMENT

Three papers reporting portions of the work presented in this thesis have been accepted for publication.

by

(a) Dixon, K.E. 1965. The ultrastructure and histochemistry of the cyst wall of the metacercaria of Fasciola hepatica L. Parasitology (in press).

K.E. Dixon

(b) Dixon, K.E. 1965. Encystment of the metacercaria of Fasciola hepatica L. in vitro. Nature, Lond., 203, 1279.

(c) Dixon, K.E. and E.H. Mercer 1965. The fine structure of the cyst wall of the metacercaria of Fasciola hepatica.

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degree of Doctor of Philosophy
in the Australian National
University. April 1965



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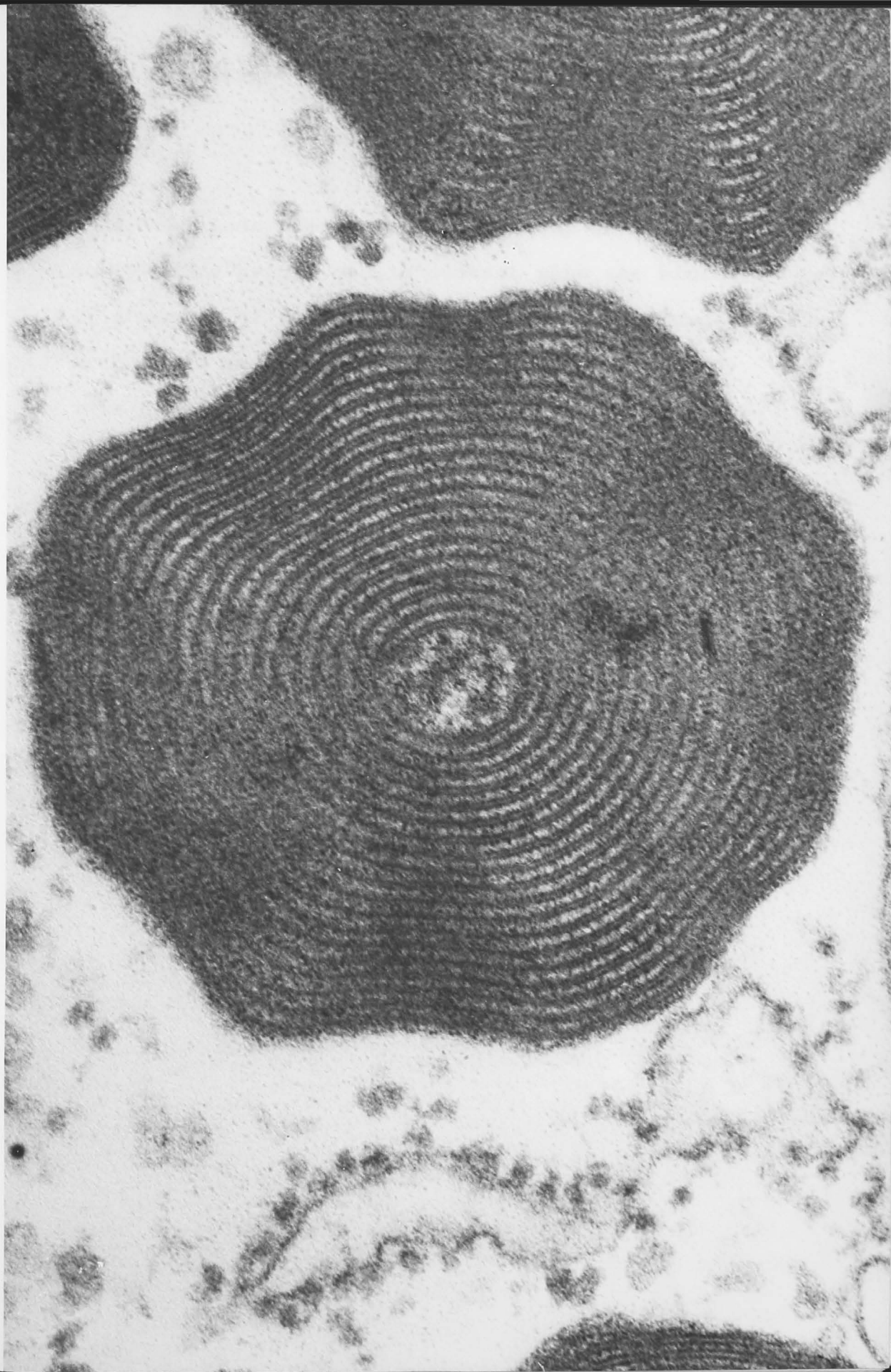
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K. E. Dixon

Frontispiece: T.S. of a keratin scroll within the
cercaria of Fasciola hepatica (x 250,000).

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"The Trematoda are a remarkable group of organisms; their study brings one in contact with the biology of a number of other groups, especially molluscs, arthropods and vertebrates which act as intermediate or definitive hosts. Furthermore, investigation of their physiology involves the use of biophysical, biochemical or immunological techniques now widely used in biological laboratories. A proper study of even a single species can thus bring the student in contact with a wide range of scientific disciplines, thereby providing an exciting introduction to fundamental biological research procedures."

SMYTH, J.D. 1965

in "The Physiology of the Trematoda".

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CHAPTER 1

INTRODUCTION: THE PROBLEM; GENERAL METHODS

The Digenea constitute one of the major groups of parasitic helminths, and their life cycles are among the most complex in the animal kingdom. It was not until 1882 that the first complete life cycle, that of Fasciola hepatica, the liver fluke of sheep and cattle, was described. By coincidence two independent investigators, Thomas and Leuckart, arrived almost simultaneously at the same conclusions. Reinhard (1957) has reviewed this work as well as the contributions of earlier investigators.

In their accounts of the life cycle, both Thomas (1883) and Leuckart (1882) described how the cercaria left the molluscan intermediate host and after swimming for some time, finally settled down on the substrate, shed its tail, and secreted a cyst wall which completely enclosed what was then the metacercaria.

It remained for Lutz (1893) to demonstrate by experimental infection that the metacercaria escapes from the cyst wall in the duodenum. Although until recent years,

F. hepatica was probably the most studied of all the digenetic trematodes, the nature of the investigations was dictated to a very large extent by immediate practical considerations. This has been in some measure responsible for a neglect of the basic biology of the parasite, so that eighty years after the pioneer work of Thomas and Leuckart, the details of most aspects of the life cycle remain obscure. In particular, almost nothing is known of the processes of infection or of the associated processes of growth and development. Therefore, the object of the investigations which form the basis of this thesis was to examine the nature of the biological processes involved in encystment and excystment of the metacercaria of F. hepatica. Initially, a study was made of the physical and chemical structure of the cyst wall. This was followed by an investigation of the mechanism of the formation of the cyst wall (encystment); for this purpose, a histological, histochemical and cytological study of cercariae at all stages of development was made. Information on the process of encystment itself, was gained from observations made on cysts at different stages of formation.

The conditions which will induce excystment of the juvenile fluke were also examined. Some information was also obtained on the methods used by the juvenile fluke to escape from the cyst wall.

Each investigation will be discussed separately, when the literature relevant to that particular aspect will also be reviewed.

MATERIALS AND METHODS

In this section, it is intended to deal mainly with those experimental procedures connected with obtaining material. The more detailed techniques are considered separately in the relevant chapters.

Materials

Lymnaea tomentosa, the molluscan intermediate host of F. hepatica in Australia, is common in the Canberra district. Snails were collected in the field and maintained in the laboratory to provide a breeding stock.

Earthenware saucers, 12 inches in diameter were used as aquaria, and to these were added pond water, sterile mud and the water weed Lemna sp. The aquaria were aerated by bubbling air through porous stones and distilled water was added periodically to offset evaporation losses. Snails were fed once weekly on an alginate based food (after Standen, 1951) in which the dried lettuce was replaced by fresh lettuce. It was found that breeding took place fairly readily in these conditions, particularly if the snails and aquaria were disturbed as little as possible.

Livers containing F. hepatica were obtained from the abattoir, the contents of the gall bladder removed and the Fasciola eggs collected by frequent sedimentation and washing. Some eggs were stored in distilled water at 4°C but usually they were cultured immediately at 26°C. The distilled water was changed periodically to control bacterial growth. After 10-12 days when the eggs contained well developed miracidia, small numbers were added to the aquaria. Approximately two months later, the snails were examined, when infected specimens could be easily identified by the presence of rediae and cercariae seen through the semi-transparent shell. When the infection was considered to be near maturity, the snails were removed from the aquaria and kept on damp filter paper in a petri dish with food available.

To collect the metacercariae, the infected snails were placed in pond water in a 100 ml beaker, which had been previously lined on the inner surface with cellophane (Urquhart, 1954). The greatest number of cercariae emerged when the snails were exposed overnight. When the emergence of cercariae had ceased, the snails were returned to the petri dishes and again exposed some time (usually 24 hr) later.

The cercariae encysted on the cellophane, which was then removed from the beaker, washed several times in distilled water and stored at 4°C over damp filter paper in sealed containers.

An outer, incomplete brown layer, the outer cyst, could be separated from the inner translucent cyst containing the metacercaria. This was most conveniently done by applying gentle pressure with, for example, the back of a microscalpel to the reverse side of the cellophane on which the metacercariae were encysted.

When immature cercariae were required free from snail tissue, an infected snail was transferred to tap water, the shell removed and the digestive gland gently teased apart to expose the rediae and cercariae.

CHAPTER 2

THE STRUCTURE AND HISTOCHEMISTRY OF THE CYST WALL OF THE METACERCARIA OF FASCIOLA HEPATICA

INTRODUCTION

In the Digenea, the most common method of infection of the definitive host is by ingestion of a free or encysted stage, the metacercaria. In most families the metacercaria occurs in the muscles or viscera of the second intermediate host, but in the Fasciolidae, Notocotylidae, Paramphistomidae, Pronocephalidae and Philophthalmidae, a second intermediate host is not required and the cercaria encysts shortly after leaving the molluscan host. In the past, some confusion in several points of terminology has arisen, and here the term "metacercaria" is used to refer to the animal which is enclosed in a cyst wall, the whole forming a cyst.

The cyst wall of Fasciola hepatica has been described as a two-layered structure (Wright, 1927; Rees, 1932; Wesenberg-Lund, 1934) although Sinitsin (1933) drew the inner cyst of F. halli (= F. hepatica) to show two layers, which with the outer cyst made up a three-layered cyst wall.

The metacercariae of other species in the Fasciolidae have been reported to have two layered cyst walls: F. gigantea (Alicata, 1938; Thapar and Tandon, 1952); F. nyanzae (Dinnik and Dinnik, 1961); Fascioloides magna (Swales, 1935) and Fasciolopsis buski (Barlow, 1925).

The metacercariae of most other species which encyst on grass or debris have been reported to have a double layered cyst wall, e.g. Paramphistomum microbothrium (Lengy, 1960), Parorchis acanthus (Rees, 1937) and Catatropis johnstoni (Martin, 1956). Singh and Lewert (1959) have demonstrated that the cyst wall of Notocotylus urbanensis consists of five concentric layers.

In most cases, those metacercariae which encyst in a second intermediate host have been reported to have a single layered cyst wall, e.g. Posthodiplostomum minimum (Hunter and Hunter, 1940). However, the metacercaria of the opisthorchid Amphimerus elongatus has been shown by Wallace (1939) to be enclosed by three separate cyst membranes. A similar complexity of structure has been observed by Feldman (1941) for Psilostomum reflexae (= Protechinostoma mucronisertulatum (Beaver, 1943)).

The chemical nature of the cyst wall of N. urbanensis has been investigated by Herber (1950) by chemical analysis but his results are difficult to interpret since he used

whole cysts and many of his techniques were not sufficiently sensitive. Singh and Lewert (1959) used histochemical tests on whole mounts and sections of this cyst and they concluded that all five layers were muco- and/or glycoprotein in nature.

Lenhoff et al (1960) have made a preliminary biochemical study of the cyst wall of a new species of Ascocotyle and they concluded that one of the components was similar to collagen.

Bogitsh (1962) has studied the chemical nature of the single layered wall of the cyst of P. minimum using histochemical techniques and Lynch and Bogitsh (1962) have confirmed these results by chemical analysis. They concluded that the wall was composed of carbohydrate-protein complexes.

MATERIALS AND METHODS

Cysts

The cysts used in this study were obtained from natural and experimental infections of snails collected in the field and from experimental infections of laboratory bred snails.

Histological Treatment

Both entire cysts and inner cysts were fixed in either 10% neutral formalin, 10% formol-saline or formol-calcium (Baker, 1960) for 24 hr to several weeks. After upgrading

to 70% ethanol, the cysts were placed in the lumen of a short length of previously fixed rat artery or small intestine (Weesner, 1960) which was then processed normally. All material was embedded in 58°C paraffin after which 7 μ sections were cut.

Infiltration of the interior of the cyst with paraffin proved impossible due to the impermeability of the cyst wall. The cysts were easier to section after Peterfi's double embedding technique (Carleton and Drury, 1957) was used but it was apparent that the wall was also impermeable to celloidin. In spite of this difficulty, the only adverse effects noted were slight distortion of the general shape and some fracture of the cysts during sectioning.

Frozen sections 10 μ thick of unfixed cysts were also cut using a freezing microtome. No distortion of the cyst was evident but in some sections the outer cyst became separated from the inner. Sections were transferred to albumen smeared slides and then dried overnight. Some were left unfixed and others were fixed immediately on transfer to the slide, in formol-calcium, 10% neutral buffered formalin or 10% formalin containing 0.5% cetyl pyridinium bromide (after Williams and Jackson, 1956).

Structure of the Cyst Wall

Observations on the general structure of the cyst wall were made on stained whole mounts of unfixed inner cysts and on unstained paraffin and frozen sections of both entire and inner cysts using phase contrast.

Partial digestion of some cysts was carried out in 0.3% crude trypsin (Nutritional Biochemicals Corporation, 4 x U.S.P. Pancreatin) and 10% sheep bile in Hank's buffered saline at 39°C for 15 min.

Histochemistry of the Cyst Wall

The histochemical reactions performed were in general those recommended by Pearse (1961), although in each case the appropriate authority is cited. The tests used are summarized in Table 2.1.

Carbohydrates

Tests for carbohydrates were made on paraffin sections and on frozen sections fixed in 10% formalin containing 0.5% cetyl pyridinium bromide. The periodic acid Schiff (PAS) reaction (after McManus, 1946) with the associated salivary amylase digestion technique was principally used. Control slides were treated with Schiff reagent without prior oxidation to check the presence of free aldehydes. A positive PAS reaction after amylase digestion indicates the presence of

1,2 glycol groups. Leblond et al (1957) consider that only carbohydrate-protein complexes are capable of giving a diastase fast positive PAS reaction. Such complexes include the neutral mucopolysaccharides and the muco- and glyco-proteins of Pearse's (1961) classification. The terminology appears to be in some confusion, particularly with regard to distinctions between those PAS positive substances which give a positive reaction with protein reactions (such as the mercury brom-phenol blue test) and those which give a negative result with such reactions. For convenience, the former substances will be termed mucoproteins since by definition this class contains more protein, and the latter neutral mucopolysaccharides, although histochemically these terms do not appear very meaningful otherwise.

Mucopolysaccharides with free acidic groups were detected with the alcian blue (AB) and ABPAS techniques of Mowry (1963). Spicer (1963) recognizes four major classes of acid mucopolysaccharides which are most easily distinguished between by their azure A extinction values and by the type of metachromasia produced with azure A or toluidin blue.

Proteins

Protein reactions were carried out on paraffin sections and on fixed frozen sections.

The mercury brom-phenol blue (HgBPB) technique (Bonhag, 1955) was used to show the general distribution of protein. The presence of disulphide bonds and sulphhydryl groups was detected using the RSR method (Bennett, 1951) and the DDD method (Barrnett and Seligman, 1954). Inner cysts were treated with 0.5 M thioglycollic acid buffered at pH 5.5 (Rogers, 1959) to reduce the disulphide bonds and then sectioned on the freezing microtome. In addition, unfixed frozen sections were treated with thioglycollic acid adjusted to pH 8. Control sections were treated with N ethyl maleimide as a block for sulphhydryl groups.

Lipids

Paraffin sections and unfixed frozen sections were used. Sudan IV in propylene glycol (after Chiffelle and Putt, 1951), Fettrot 7B in acetone/70% ethanol (Gurr, 1958) and oil red O (after Lillie, 1944) were used. A positive reaction implied the presence of neutral fats. Overnight extraction with hot chloroform-methanol was used as a control.

Solubility Tests

Brown (1950) has studied the solubility in different reagents of proteins bonded by various linkages. These reagents were used on outer and inner cysts in order to define the linkages present.

Polarized Light Studies

Unstained paraffin sections and frozen sections were examined under polarized light in an attempt to detect oriented anisotropic structures in the cyst wall.

OBSERVATIONS AND RESULTS

1. General Morphology

Whole Mounts: From a lateral view, the inner cyst has a roughly hemispherical shape and is covered dorsally and laterally by the outer cyst which has a flared base where it is attached to the substratum (Figs. 2.4; 2.6). Viewed from above the inner cyst usually appears circular in shape (Fig. 2.1) but occasionally appears oval shaped or roughly figure-of-eight shaped.

Of the 15 vital dyes used only toluidin blue produced differential staining. Observations of inner cysts stained overnight in 0.01% toluidin blue showed that the inner cyst wall was composed of two major layers, an outer structureless pink layer showing slight γ metachromasia, and an inner relatively thick, coarsely laminated dark blue layer. Closely apposed to the inner surface of this laminated layer was a thin, bright blue band which enclosed the purple staining cyst fluid and the metacercaria (Fig. 2.5). This band is not considered to be a separate layer (see below). After

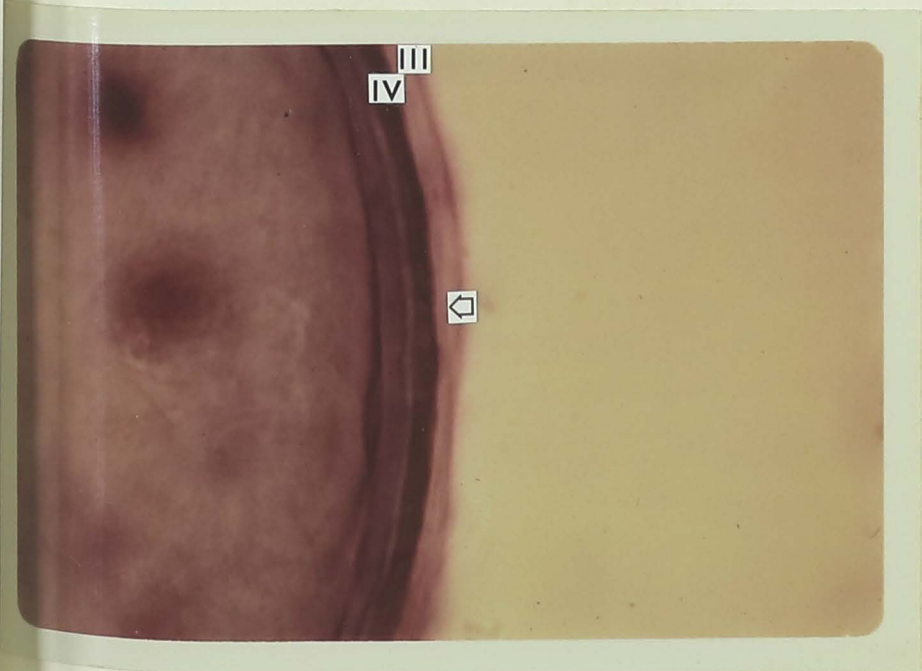
Fig. 2.1: Whole mount of inner cyst; the structureless Layer III is external to the darker Layer IV. The suckers and the concretions in the excretory system are also visible.

Fig. 2.2: Toluidin blue stained whole mount of inner cyst after partial digestion with trypsin and bile; Layer III has expanded to show the metachromatically stained band III b.

Fig. 2.3: The thickened area of the ventral plug has slightly different optical properties. Elsewhere in Layer IV there are suggestions of the lamellar appearance which this layer shows.

Fig. 2.4: Section of the entire cyst stained with the ABPAS procedure. Note the flared base of Layer I and the ABPAS positive Layers II and III.

Fig. 2.5: Toluidin blue stained whole mount of inner cyst. Layers III and IV are visible and within the former, the metachromatic Layer III b is prominent (arrow).



partial digestion with trypsin and bile, the structureless outer layer expanded considerably and toluidin blue staining revealed a strong γ metachromatic band in the middle of this layer (Fig. 2.2).

Sections: A phase contrast study of unstained paraffin and frozen sections revealed the same general shape and structure as was seen in the whole mounts except that no subdivision of the structureless outer layer of the inner cyst could be detected. In the ventral region, the central portion of the laminated layer appeared much thicker and less dense than elsewhere (Figs. 2.3; 2.6). The outer cyst was seen to be formed of two distinct divisions, a thick granular outer layer with a thin, slightly fibrous layer on the inner surface (Fig. 2.6).

In all, four major layers could be distinguished making up the cyst wall and for convenience these are numbered I to IV, layer I being outermost (Fig. 2.6). The subdivisions of the structureless layer of the inner cyst are designated III a, b and c respectively, III a being the outer layer.

2. Histochemical and Related Tests

The results of the histochemical tests are summarized in Table 2.1. For convenience, each layer will be discussed separately.

Layer I: The thick outermost cyst membrane was positive only with the HgBPB reaction indicating a protein composition. It was soluble only in sodium hypochlorite (Brown, 1950) and this coupled with the brown colour of the cyst which darkens with age strongly suggests that this layer is composed of a tanned protein.

Layer II: After the ABPAS reaction this layer appeared to be composed of purple and red strands (Fig. 2.4). It was concluded that this layer is made up of strands of mucoprotein and acid mucopolysaccharide.

Layer III: The distribution and relative widths of the subdivisions of this outermost layer of the inner cyst varied slightly between specimens. The most consistent results were obtained from frozen sections post fixed with 10% formalin containing 0.5% cetyl pyridinium bromide or with 10% neutral buffered formalin. Dorsally, the appearance was similar to that observed in the whole mounts but in the lateral regions III a became relatively much thinner and was usually completely absent ventrally where III c was much thicker.

The ABPAS reaction gave reasonably consistent results, as shown in Table 2.1. With aqueous toluidin blue, III b showed strong γ metachromasia. Results with the HgBPB test

Table 2.1: Results of histochemical tests on the cyst wall

Histochemical Reaction	Cyst layers						Ventral Plug
	I	II	IIIa	IIIb	IIIc	IV	
PAS	-	+	+	+	+	-	+
PAS after diastase	-	+	+	+	+	-	+
Schiff	-	-	-	-	-	-	-
AB	-	+	-	+	-	-	-
Azure A pH 0.6	-	+	-	+	-	-	-
Toluidin Blue metachromasia	-	+	-	+	-	-	-
HgBPB	+	+	+	-	-	+	-
RSR	-	-	-	-	-	-	-
RSR after reduction with thioglycollic acid	-	-	-	-	-	+	-
RSR after reduction followed by blockage with N-ethyl maleimide	-	-	-	-	-	-	-
DDD	-	-	-	-	-	-	-
DDD after reduction with thioglycollic acid	-	-	-	-	-	+	-
DDD after reduction followed by blockage with N-ethyl maleimide	-	-	-	-	-	-	-
Sudan IV	-	-	-	-	-	-	-
Fettrot 7B	-	-	-	-	-	++	-
Fettrot 7B after extraction	-	-	-	-	-	-	-
Oil red O	-	-	-	-	-	++	-
Oil red O after extraction	-	-	-	-	-	-	-

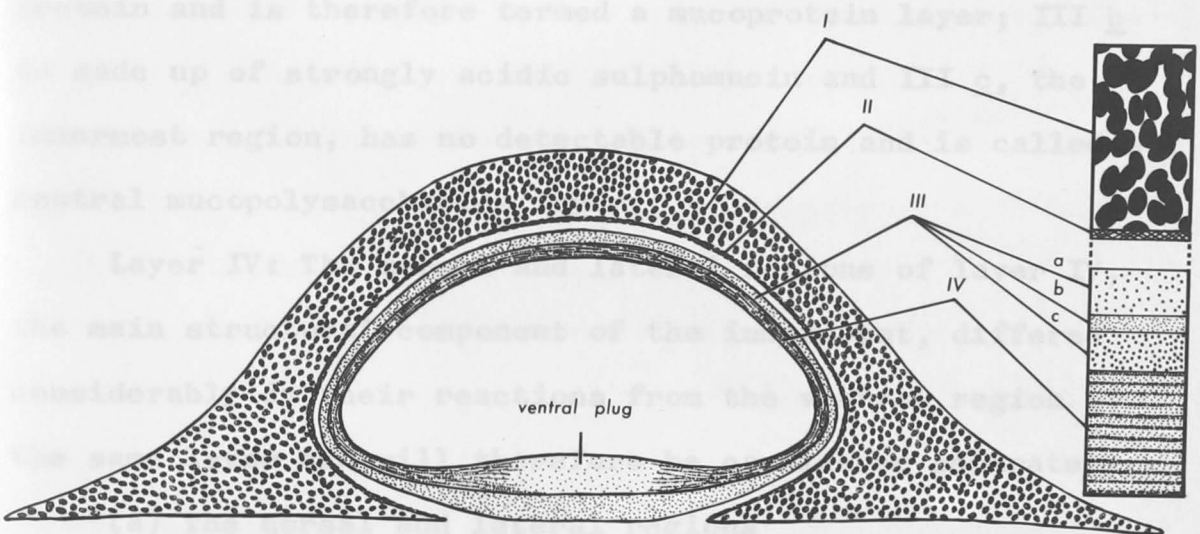
* positive in part only - see text.

Fig. 2.6: A. Diagrammatic representation of the structure of the cyst wall of the metacercaria of F. hepatica in vertical section showing the relationships of the Layers I-IV and the ventral plug.

B. An enlargement of part of the dorsal side of the cyst wall, showing in particular the subdivisions of Layer III.

more variable but in general III a was positive and
 III b and c were negative.

These results indicate that layer III is composed of
 carbohydrate-protein complexes, the relative proportions of
 carbohydrate and protein differing in various regions. The
 innermost region III a contains a higher proportion of
 protein and is therefore termed a mucoprotein layer; III b
 is made up of strongly acidic sulphomucopolysaccharides. The
 outermost region, has no detectable protein and is called
 central mucopolysaccharide layer.



Tests for carbohydrates were uniformly negative. The
 PAS reaction produced a strong reddish blue colour and in
 places where the lamellae were separated, a light blue
 colour was seen. On the inner and outer edge of this layer
 a thin light blue fringe could often be detected. On the
 inner edge this corresponded to the innermost light blue
 band seen in the toluidin blue stained whole mounts.

were more variable but in general III a was positive and III b and c were negative.

These results indicate that layer III is composed of carbohydrate-protein complexes, the relative proportions of carbohydrate and protein differing in various regions. The outermost region III a contains a higher proportion of protein and is therefore termed a mucoprotein layer; III b is made up of strongly acidic sulphomucin and III c, the innermost region, has no detectable protein and is called a neutral mucopolysaccharide layer.

Layer IV: The dorsal and lateral regions of layer IV, the main structural component of the inner cyst, differed considerably in their reactions from the ventral region of the same layer and will therefore be considered separately.

(a) The dorsal and lateral regions

Tests for carbohydrates were uniformly negative. The HgBPB reaction produced a strong reddish blue colour and in places where the lamellae were separated, a light blue colour was seen. On the inner and outer edge of this layer a thin light blue fringe could often be detected. On the inner edge this corresponded to the innermost light blue band seen in the toluidin blue stained whole mounts.

The DDD reagent produced a strong purple reaction with thioglycollate treated material indicating the presence of a large number of disulphide bonds.

Unfixed frozen sections stained with Fettrot 7B gave a positive reaction on the inner and outer surfaces of layer IV. The colour produced faded quickly towards the interior of the layer. A similar but weaker reaction was obtained with oil red O. Control slides were negative and paraffin sections were not stained by these dyes and this latter result may be due to extraction of the lipid fraction during histological processing.

When whole inner cysts were treated in the reagents of Brown (1950), this layer was only affected by thioglycollic acid and sodium hypochlorite in both of which it dissolved. It was resistant to the enzymes pepsin, trypsin, lipase, α amylase, pronase and bacterial collagenase.

Under polarized light layer IV showed strong negative birefringence which appeared to be uniaxial. The results were similar for paraffin sections and for frozen sections. Birefringence of this type could be produced by a series of lamellae parallel to the edges of the layer.

From these results it would appear that there are two components of the dorsal and ventral part of layer IV, a protein stabilized by disulphide linkages forming lamellae

which are embedded in a protein-lipid matrix which can be seen only at the edges or where the lamellae are separated.

(b) The ventral region

The thickened central portion of the ventral side of layer IV gave a positive PAS reaction. The HgBPB reaction produced a yellow brown colour in this area. In 0.2 N sodium hydroxide, solution occurred within 1 hr. This was a similar reaction to that shown by layer III and indicates a similarity in their chemical constitution. It would appear from this evidence that the ventral plug, as it may be conveniently termed, is composed of neutral mucopolysaccharide.

DISCUSSION

The results of this study indicate that the cyst wall of the metacercaria of F. hepatica is made up of four major layers. Their chemical composition as determined from histochemical reactions is as follows:

Layer I - tanned protein

Layer II - mucoprotein and acid mucopolysaccharide

Layer III (a) - mucoprotein

(b) - acid mucopolysaccharide

(c) - neutral mucopolysaccharide

Layer IV, dorsal and lateral regions - keratinized protein
embedded in a matrix consisting of protein
and lipid.

ventral plug region - neutral mucopolysaccharide.

The complexity of the cyst wall of F. hepatica metacercariae demonstrated in this investigation suggests that further study of cysts of other species using histochemical techniques would reveal a similar complexity of structure.

The obvious function of the cyst wall is to provide protection for the metacercaria in its resting stage as well as during its passage to the site of excystment in the final host. It might be expected therefore, that the cyst walls of free encysting metacercariae would be more complex than those of metacercariae encysting in a second intermediate host. The observations already reported would appear to support this view, although most studies have not been sufficiently critical to provide a reliable comparison.

In the resting stage, protection against desiccation, against mechanical injury, against toxic substances and against attack by other organisms is required. Shirai (1927) has tested the resistance of F. hepatica cysts to some of these factors. He has shown that the cysts are still infective after being dried for 24 hr. They are also able to resist the effects of 0.1% corrosive sublimate solution for 24 hr., 50% alcohol for 2 hr. and 0.8% formalin or 3% saline for 24 hr.

The outer tanned protein layer probably acts as a barrier against bacterial and fungal attack (W.L. Nicholas, personal communication) since tanned proteins are among the most resistant substances known to occur. It is likely that the metacercarial cyst wall of paramphistomes has an outer tanned protein layer also, since the cysts are usually described as dark brown in colour and Durie (1953) has remarked that the cyst of Ceylonocotyle streptocoelium darkens with age which is a characteristic of tanned materials.

It is difficult to ascribe a function to the mucopolysaccharide layers II and III although it would seem that these substances commonly occur in cyst walls (Singh and Lewert, 1959; Bogitsh, 1962). The central position of the acid mucopolysaccharide layer III b does not appear merely fortuitous although the reason for such an arrangement is obscure.

Layer IV consists of protein stabilized by disulphide linkages, which may be called a keratin, held in a protein-lipid matrix. According to Baldwin (1937), vertebrate keratin originally functioned as a water proofing element and here it could provide some protection against desiccation, in particular since its lamellar structure would be well suited to this function. It is likely that the lipid part of the matrix is also important in this regard. Keratin

is moreover, also highly resistant to enzymatic action and thus provides additional protection against other organisms and against the digestive enzymes of the host. This layer as a whole is most important in providing structural rigidity. This is easily demonstrated by attempting to pierce the inner cyst when considerable pressure is required.

The ventral plug region contains carbohydrate-protein complexes and its structure will be further discussed in Chapter 3. During excystment, the metacercaria emerges through a hole in the cyst wall formed by digestion of this region. The ventral plug may thus be considered as a specially weakened area of the cyst wall.

CHAPTER 3

THE FINE STRUCTURE OF THE CYST WALL OF THE METACERCARIA OF FASCIOLA HEPATICA

INTRODUCTION

The cyst wall which encloses the metacercaria of Fasciola hepatica has been studied using histochemical techniques and conventional light microscopy (Chapter 2). An outer tanned protein layer and an inner carbohydrate-protein layer form the outer cyst which may be removed mechanically. The inner cyst is composed of an outer carbohydrate-protein layer in which may be distinguished several sub-layers differing in their chemical characteristics, and an inner keratinized layer stabilized by disulphide linkages and held in a protein-lipid matrix. A further investigation was carried out with the electron microscope with the object of elucidating the fine structure of the cyst wall.

MATERIAL AND METHODS

Both the entire cyst and the inner cyst, obtained by mechanically removing the outer wall, and some cysts with partly formed walls, were used in this study.

1. Preparation for Microscopy

It has been noted (Chapter 2) that the cyst wall is virtually impermeable to reagents, and therefore, the cysts were punctured with a microscalpel or razor blade as quickly as possible after transfer to the fixative. This procedure facilitated the penetration of the fixative and the infiltration of the embedding material. Some cysts were treated in 0.5 M thioglycollic acid in acetate buffer at pH 5.5 (Rogers, 1959) to reduce the disulphide linkages of the keratinized layer.

(a) Fixation: Standard buffered (pH 7.2) 1% solutions of osmium tetroxide or Baker's formol-calcium (Baker, 1960) were used. Cysts were fixed in the osmium solution for 2-12 hr. and in the formol-calcium for 24 hr. After fixation the cysts were rinsed in tapwater and dehydrated in successive changes of 70% and absolute ethanol, each of 1 hr. Infiltration was carried out in a mixture of equal parts of araldite and ethanol followed by pure araldite, both at 60°C for 1 hr. The cysts were finally embedded in a further change of

araldite and allowed to harden at 60°C for 48 hr. Sections, passing roughly through the centre of a cyst, were cut on a LKB microtome using a diamond knife and were mounted on copper grids without a supporting film.

(b) Staining: Sections were stained by floating grids on staining solutions for 30 min. at 40-50°C. Good results were obtained with lead solutions (Karnovsky, 1961; Millonig, 1961) and use was also made of 1% solutions of either uranyl acetate or potassium permanganate.

2. Electron Microscopy

Sections were studied and photographed in an Elmiskop 1 electron microscope at instrumental magnifications of 2,500-40,000.

RESULTS AND DISCUSSION

No difficulty was experienced in recognizing from their locations the main layers of the cyst wall, and these are shown in Fig. 3.1, a low power survey micrograph.

1. The Outer Cyst Wall

(a) The tanned layer (I): In the dorsal and lateral regions, the external layer of tanned protein is the thickest layer, and has the most open texture (Fig. 3.1). It consists of spongey aggregations of dense, irregularly shaped bodies.

Closer examination of sections cut in different directions confirmed that these aggregations consist of numbers of dense, elongated, (cigar-shaped, objects apparently held together in a matrix of somewhat less dense material (Fig. 3.2). Their appearance is essentially the same in unstained material (although less apparent) as in material stained with uranium or lead. The surfaces of these irregular masses fuse when they are in contact, giving the impression of being sticky. The entire layer, however, in no place appears compact, numerous interconnecting cavities remaining. The impression gained is that it would be rather porous, its value to the encysted animal being to add to the mechanical protection and perhaps to act as a barrier to bacterial and fungal penetration (see Chapter 2).

(b) The carbohydrate-protein layer (II): This layer appears as a feltwork of very fine, poorly staining filaments (Fig. 3.1). No structural distinction was observed between the parts of the layer known to give reactions of acid mucopolysaccharide and mucoprotein. These reactions presumably are due to chemical end-groups distributed in varying amounts in a structural framework of macromolecular dimension which can be seen in the electron microscope.

2. The Inner Cyst Wall

(a) The carbohydrate-protein layer (III): On the ventral surface of the cyst, this layer appears relatively homogeneous, but on the lateral and dorsal surfaces, it may be resolved into an outer slightly denser layer, with a finely granular appearance, and an inner structureless layer. The outer one is thought to be the mucoprotein layer III_a (Chapter 2). The inner is made up of acidic and neutral mucopolysaccharide (Chapter 2) and these chemical differences may correspond to the structural differences evident in Fig. 3.3.

(b) The keratinized layer (IV): Of the major layers, this one is the most distinct, compact and dense. At low power (Fig. 3.1) it appears homogeneous except for an occasional splitting into sheets, an effect also visible in the light microscope where the appearance suggests that this layer is coarsely laminated.

At higher powers (Fig. 3.4) the formation is resolved into a succession of tightly compacted, fine, uniform lamellae each about 75A in thickness and separated by less dense layers of similar dimensions composed of a finely particulate material. This structure is usually very regular but some separation of the fine lamellae is occasionally apparent and other defective areas are not uncommon

(Fig. 3.1). Some of these may have resulted from the process of formation (see Chapter 4).

In wider gaps between the lamellae the less dense amorphous substance sometimes appears in larger amounts, particularly near the outer surface of the entire formation. This inter-lamellar component may conceivably function as an adhesive holding together the sheets of the lamellar component. It may correspond to the protein-lipid matrix of this layer (Chapter 2) but there is no method by which its composition could be determined at this level.

In sections of cysts pretreated with buffered thio-glycollate solution, the lamellae are more widely separated (Fig. 3.5). This suggests that the disulphide bonds occur predominantly between the lamellae. At higher magnifications each lamella appears as a double strand.

This structure has some resemblance to the keratinized formations of the vertebrate epidermis. Vertebrate keratin is recognized to contain two components: a fine fibrillar material (α -keratin) and an amorphous matrix (γ -keratin), which are closely bonded together by the formation of disulphide bonds mainly located in the γ -component (Mercer, 1961). In the cyst wall we find fine sheets presumably bonded by a second component (see Chapter 4).

Superficially the keratinized layer of the cyst wall recalls the myelin sheath of myelinated nerves and the spherulites formed in water by phospholipids, although of course, there is no chemical similarity. A structural distinction is the presence of 'free ends' in cross sections of the keratinized lamellae. Such ends are almost never found in myelin formations.

(c) The ventral plug: The ventral plug region is difficult to define with certainty under the electron microscope. Sections through this region show lamellae similar to those seen elsewhere in the keratinized layer, but in this case forming a far less compact region. Whereas in the dorsal region of the keratinized layer, individual lamellae are difficult to distinguish under the highest magnification, in the ventral plug region individual lamellae are widely separated and easily distinguished.

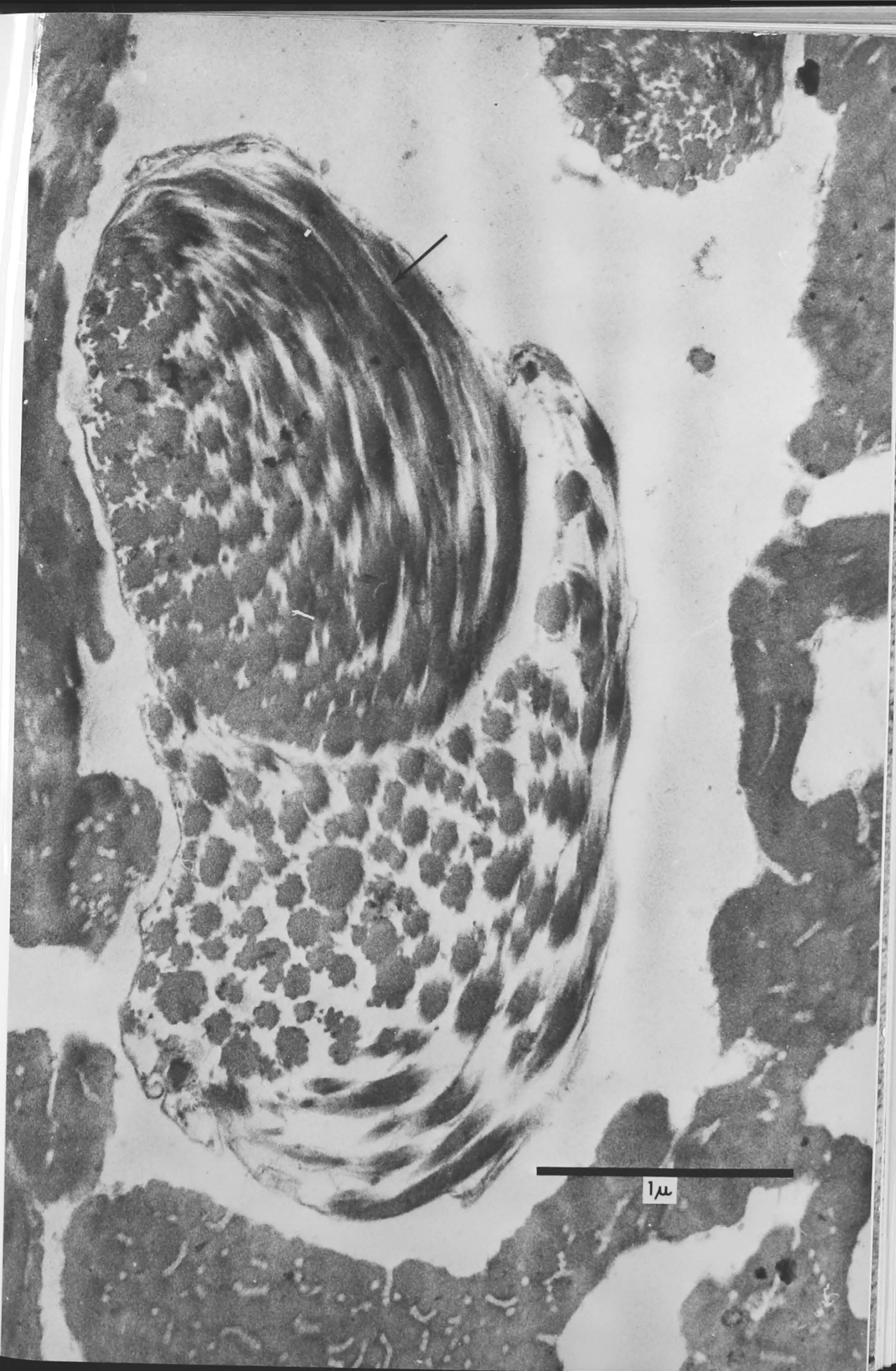
Fig. 3.1: Electron micrograph. Section through cyst wall to show the relationships of the layers. The roman numerals refer to the layers of the cyst wall. In Layer I, the cigar-shaped filaments are cut in cross section. II is thicker in this region than usual and IIIa, the mucoprotein layer of the inner cyst wall cannot be distinguished. The dense nature of Layer IV, the keratinized layer of the inner cyst wall is evident. The coarse lamellae (arrow) would be visible in the light microscope. Some imperfections (k) are apparent. Osmium tetroxide, Uranyl acetate.



Fig. 3.2: Electron micrograph. Portion of the tanned outer Layer I of the cyst wall. The cigar shaped particles (arrow) are visible and elsewhere the meshwork, formed by the adherence of adjacent granules is evident. Osmium tetroxide, Uranyl acetate.

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Fig. 3.3: Electron micrograph. The carbohydrate-protein Layer III at high magnification. IIIa has a more open texture than the rest of the layer. The acid mucopolysaccharides may be represented by the more fibrous components (arrow). Osmium tetroxide, Millonig's lead.

IIIa

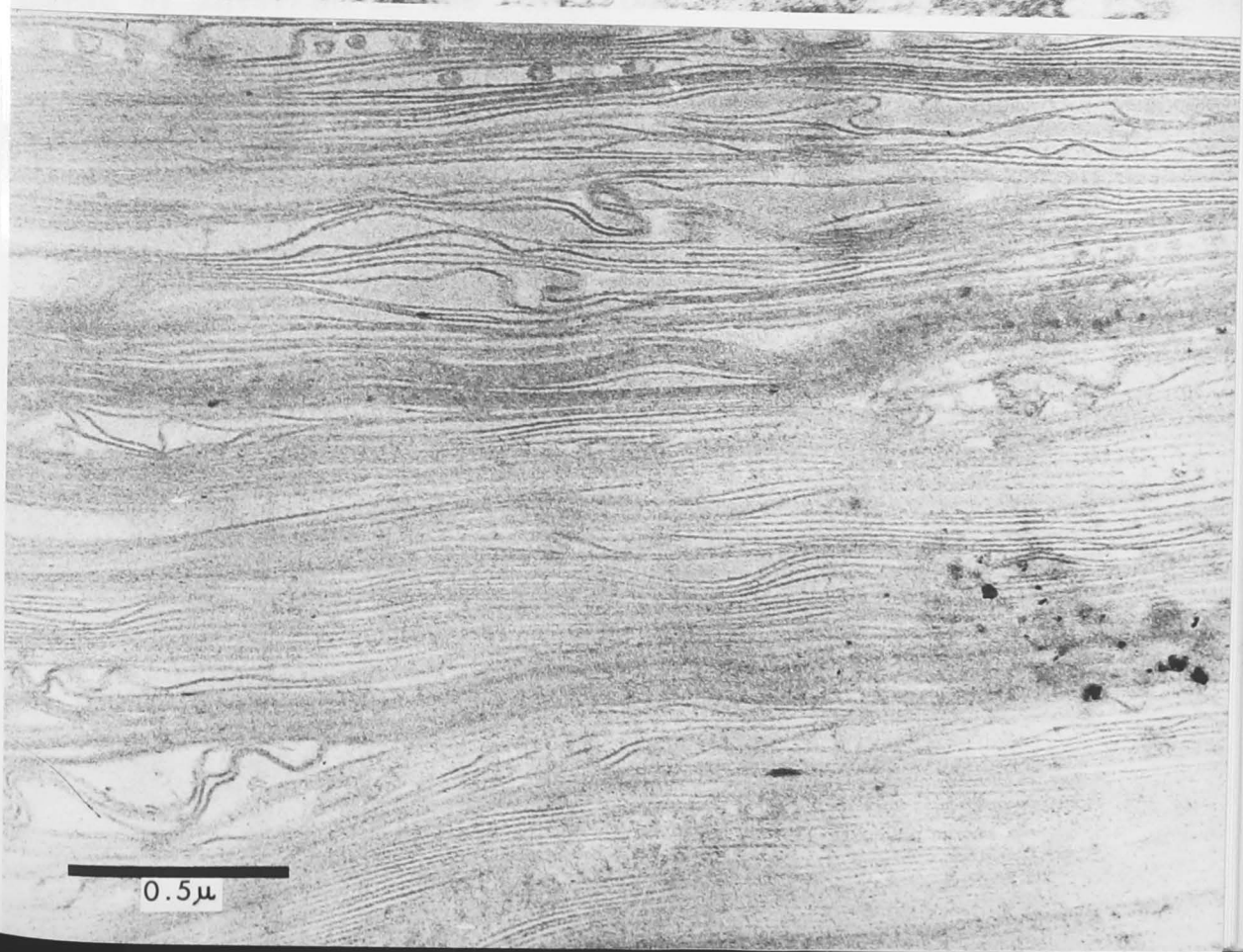
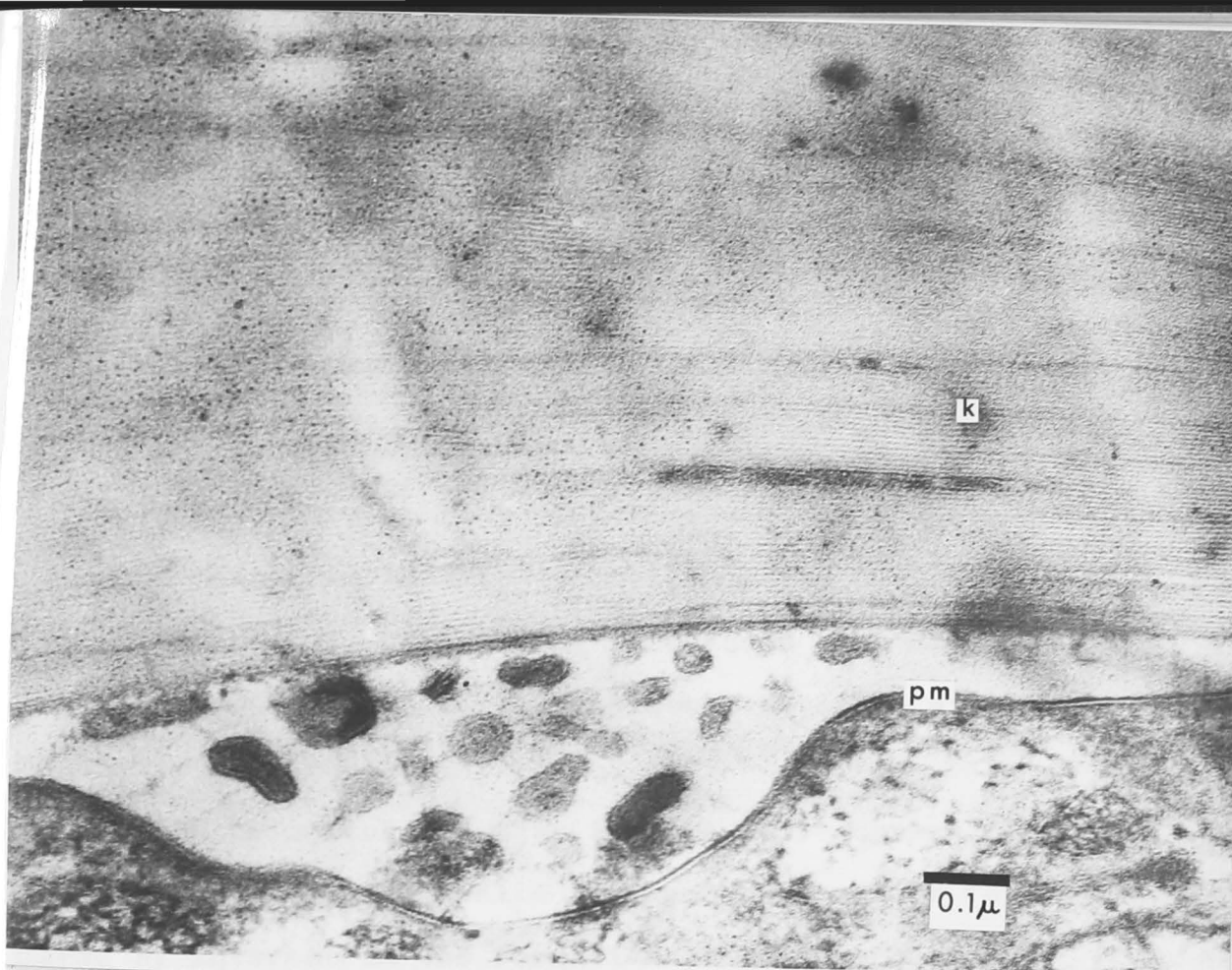
III



0.1μ

Fig. 3.4: Electron micrograph. View at high magnification of the keratinized Layer IV of the cyst wall (k) which is composed of numerous closely packed lamellae. The plasma membrane (pm) indicates the edge of the metacercaria. Osmium tetroxide, Karnovsky's lead.

Fig. 3.5: Electron micrograph. The appearance of the cyst wall after treatment with thioglycollic acid. The lamellae are more widely separated than in Fig. 3.4. Osmium tetroxide, Millonig's lead.



CHAPTER 4

THE ENCYSTMENT OF THE CERCARIA OF FASCIOLA HEPATICA

It has been shown that the cyst wall of the metacercaria of F. hepatica is a complex structure and it has been suggested in Chapter 2 that investigations into the structure of other metacercarial cysts using techniques such as histochemistry and electron microscopy could reveal a similar complexity of structure.

It can be speculated that failure to recognize the complicated nature of cyst walls has been responsible for the dearth of interest in the formation of these structures. Many of the observations on the encystment process have been made only on living material. Stirewalt (1963) for example, has paraphrased Rees' description of the encystment of the cercaria of Parorchis acanthus as follows:

First, the encysting cercaria attaches to an appropriate spot by the ventral sucker, and mucous, which sticks the cercaria to the substratum, is secreted by conspicuous ventral glands through pores in the cuticle. Then the body contracts, the oral sucker attaches, and mucous from anterior glands pours over it, again cementing it to the substratum. In turn, mucous flows out, this time through dorsal cuticular pores from dorsal glands to cover the entire larva with a thin film. This is the mold in which

cystogenous materials are cast in the specific cyst form. Into this mold granules stream out of ventral cystogenous cells and are pressed by the body against the mucous film to form an outer margin of the cyst. More cystogenous material is secreted, and forced up by the cercarial body to form the dome of the cyst. Opposite the oral sucker the cyst is temporarily incomplete, but the sucker is retracted into the body and the paired cystogenous gland termini are brought together, and these operate to seal the opening. The tail is forced away and severed, leaving only a minute indentation to mark the spot where the secretion hardened while the tail attached. After the cystogenous secretion has been completely emitted, the cercariae actively smooth the still plastic material inside. Later an inner membrane is secreted, probably akin to the original mucous secretion. The latter forms a thin elastic inner wall...This entire process has been observed to occur within 10 minutes.

To investigate encystment, it is first of all necessary to study the histology of the cercaria, particularly in relation to the secretory cells. Proceeding from a knowledge of the structure of the cercaria, it is then possible to analyse the encystment process by examining stages in the formation of the cyst wall.

Thus, observations made on sectioned material by Wright (1927), Bovien (1934) and Wesenberg-Lund (1934) suggested that studies of encystment made solely on the living cercaria of F. hepatica would be inadequate. Rothschild (1936) and Rees (1937) also studied encystment using histological techniques and made significant contributions towards an understanding of this process.

In this investigation, the histology and cytology of the secretory glands of the cercaria were investigated using histochemistry and electron microscopy. The process of encystment was then studied by combining observations on the behaviour of living cercariae and metacercariae with histological and cytological studies on cysts in different stages of development. In this way, an attempt was made to overcome the shortcomings of studies made solely on fixed material from which the course of dynamic processes can only be deduced.

MATERIAL AND METHODS

The material used in this investigation was obtained from experimental infections of laboratory reared snails.

Light Microscopy

Naturally emerged cercariae and developing cercariae within rediae were fixed, embedded and sectioned in a similar manner to that described in Chapter 2. It was found more convenient to study the developing cercariae by processing entire infected snails and thus observations could be made on cercariae in all stages of development.

Some of the sections were stained with Mallory's triple stain (Carleton and Drury, 1957) and others with the Mallory-Heidenhain rapid stain (Humason, 1962). The

histochemical reactions which were used have been described in Chapter 2. In addition, the diazo reaction to detect phenols and the catechol reaction for polyphenol oxidase (Smyth, 1954) were used.

Encystment was also studied in living material using normal, dark field and polarized illumination. A 16 mm. cine film was made to facilitate an accurate analysis of the complete process.

Electron Microscopy

Naturally emerged cercariae, free cercariae in the snail tissues and cercariae within rediae were examined, as well as cysts in various stages of formation. The intra-molluscan stages were obtained by dissecting away the shell of an infected snail and transferring the cercariae and rediae which were exposed, as quickly as possible to the fixative.

Buffered osmium tetroxide solution was the main fixative used but the time of fixation was increased to 18 hr. It has been found that cytoplasmic membranes are often more easily demonstrated after long periods of fixation (Mercer and Birbeck, 1961). Some material was also fixed in buffered 2% potassium permanganate solution for 2 hr.

The general procedures used for embedding, sectioning and 'staining' have been described in Chapter 2. Some

sections were also post-stained with a lead citrate solution (Reynolds, 1963).

OBSERVATIONS AND RESULTS

1. The Histology and Cytology of the Secretory Cells

(a) Light microscopy

Examination of sections of infected snails stained with Mallory's triple stain or the Mallory-Heidenhain rapid stain, revealed four major types of glandular cells (Figs. 4.1; 4.2). For reference purposes, these cells will be called - (a) the tanned protein cells, (b) the mucopolysaccharide cells, (c) the mucoprotein cells and (d) the keratin cells. These terms denote the histochemical characteristics of the cells and these can be correlated with the histochemical characteristics of individual layers in the cyst wall, although the evidence for this is presented later.

The tanned protein cells (tp) are concentrated in the ventral half of the body and are completely filled with large yellow-brown secretory granules.

The mucopolysaccharide cells (pc) are distributed around the periphery of the dorsal surface of the cercaria and also around the ventral sucker, in groups of one and two cells. There are approximately 50 of these gland cells on the dorsal and dorso-lateral surface, roughly arranged in

about seven or eight rows of seven cells each. Around the ventral sucker there appears to be another five or six cells. With Mallory's triple stain, these cells show little or no staining reaction; at best, a faint blue colour in the cytoplasm results.

The mucoprotein cells (mp) consist of an elongated group of about ten or twelve cells occupying a central position immediately dorsal to and slightly anterior of the ventral sucker and stretching to about the level of the pharynx, where the most anterior gland cells are close to the ventral surface. With Mallory's triple stain, these cells are very obvious because of their bright blue colour. At higher magnifications, it was seen that the colour is due to a large number of densely packed ovoid granules in the cytoplasm. Although these cells occupy a similar position to the "penetration glands" described from other cercariae (see Hyman, 1951, p. 260), ducts leading from these cells could not be distinguished.

The keratin cells (kc) occupy almost the whole of the dorsal half of the body and stain a greyish-brown colour with Mallory's triple stain. They are characterised by the presence of a large number of rod-shaped secretory particles, approximately 5μ in length, lying parallel to one another and arranged roughly in cubical "packets" approximately

5 μ square in section. The groups of rods are distributed at random through the cytoplasm.

The distribution of all these cells does not change markedly before encystment begins, with the exception of the tanned protein and keratin cells. Usually before the cercaria leaves the redia, and always before it leaves the snail, the contents of the tanned protein cells are secreted to the exterior to form a thick layer around the body of the cercaria (Figs. 4.3; 4.27). Some granules of the mucopolysaccharide cells are also extruded at about this time to lie on the surface of the cercaria. Some of the rod-containing cells then move to occupy the ventral region previously occupied by the tanned protein cells, so that the keratin cells become less closely packed.

(b) Histochemistry

The tanned protein cells

In sections stained with aqueous BPB, of cercariae within snails, the granules of the tanned protein cells were stained selectively, demonstrating that their contents consist of basic protein (Fig. 4.4).

A positive result for the presence of phenols was obtained when the diazo reaction was applied to frozen sections, the cells staining a bright orange-brown colour. The catechol reaction to detect polyphenol oxidases was negative

when used on whole cercariae or on sections fixed in 70% ethanol for periods of 2-24 hr. and also after fixation in formalin for 4 hr., 24 hr. or several days. According to Clegg and Smyth (1965) phenolase is an extremely labile enzyme in some species.

Smyth (1954) considers that three precursors are necessary for the tanning process: (a) basic protein, (b) phenols and (c) polyphenol oxidase. In this case the presence of the first two substances has been demonstrated but the presence of polyphenol oxidase is uncertain. There is no doubt, however, that the contents of the tanned protein cells form layer I of the cyst wall. Solubility experiments and changes in colour (Chapter 2) have indicated that this layer is probably composed of a tanned protein, and since two of the precursor substances have been demonstrated, it seems reasonable to conclude that this substance is, in fact, a tanned protein.

The mucopolysaccharide cells

With the PAS reaction, the contents of these cells gave a positive result which was not affected by prior digestion with saliva (Fig. 4.5). The colour produced in the cells was due to a large number of PAS positive granules in the cytoplasm. With the ABPAS procedure, some of these granules stained deep bluish-purple (Fig. 4.6). The results of

treatment with the HgBPB reagent were difficult to assess but it was concluded that the reaction was negative. The PAS positive granules are probably composed of neutral mucopolysaccharide in the restricted sense used in Chapter 2, and the ABPAS positive granules of acid mucopolysaccharide.

It seems reasonable to conclude that the contents of these cells are used in forming part of layer II of the outer cyst and in forming layers III_b and III_c of the inner cyst.

The mucoprotein cells

The granules in the cytoplasm of these cells gave a positive PAS reaction which was diastase fast. No additional staining resulted from application of the ABPAS procedure (Figs. 4.6; 4.7). A moderately positive reaction was given by the HgBPB test and on these grounds it was concluded that these cells contain mucoprotein, as defined in Chapter 2.

Layer III_a of the inner cyst wall is composed of mucoprotein and it appears probable that the contents of the mucoprotein cells form this portion of the cyst wall.

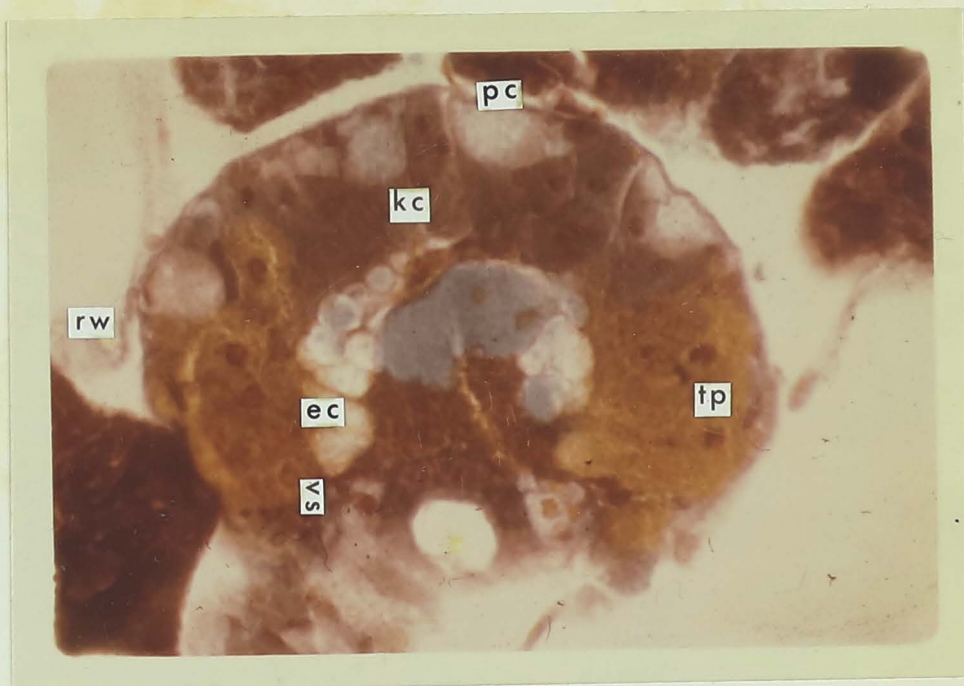
The keratin cells

The rods contained within these cells gave a positive result with the HgBPB reagent. The RSR and DDD tests for sulphhydryl groups were also positive but the colour became more intense if the sections were pretreated with thioglycollic acid. If N-ethyl maleimide which blocks SH groups was

Fig. 4.1: Light micrograph. T.S. cercaria within redia at level of ventral sucker (vs). tp, tanned protein cells; pc, mucopolysaccharide cells; mp, mucoprotein cells; kc, keratin cells; rw, redia body wall; ec, excretory canal. Mallory's triple stain.

Fig. 4.2: Light micrograph. L.S. cercaria within redia. os, oral sucker; ta, tail; ca, caecum; ec, excretory canal; tp, tanned protein cells; pc, mucopolysaccharide cells; mp, mucoprotein cells; kc, keratin cells; rw, redia body wall. Mallory's triple stain.

mp



tp

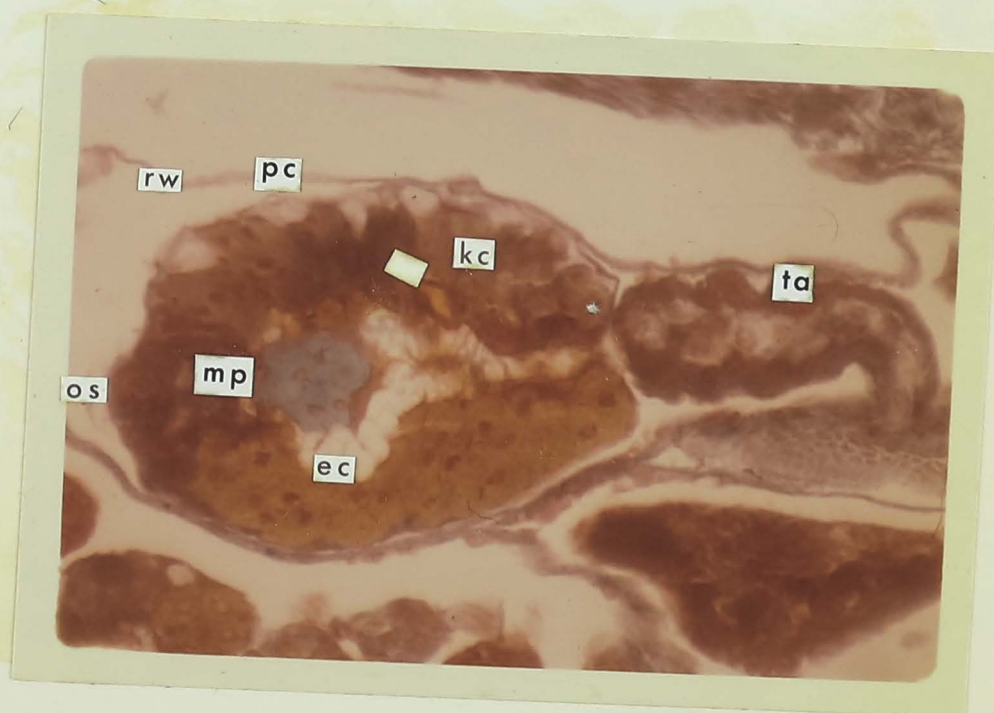


Fig. 4.3: Light micrograph. T.S. free swimming cercaria with a layer of tanned protein material (t) enclosing the body of the cercaria. The keratin cells (kc) have moved into the region formerly occupied by the tanned protein cells. The mucoprotein cells (mp) are visible. ca, caecae. Mallory-Heidenhain rapid.

Fig. 4.4: Light micrograph. T.S. cercaria within a redia stained with aqueous BPB. The tanned protein cells (tp) are selectively stained. BPB, Neutral red.

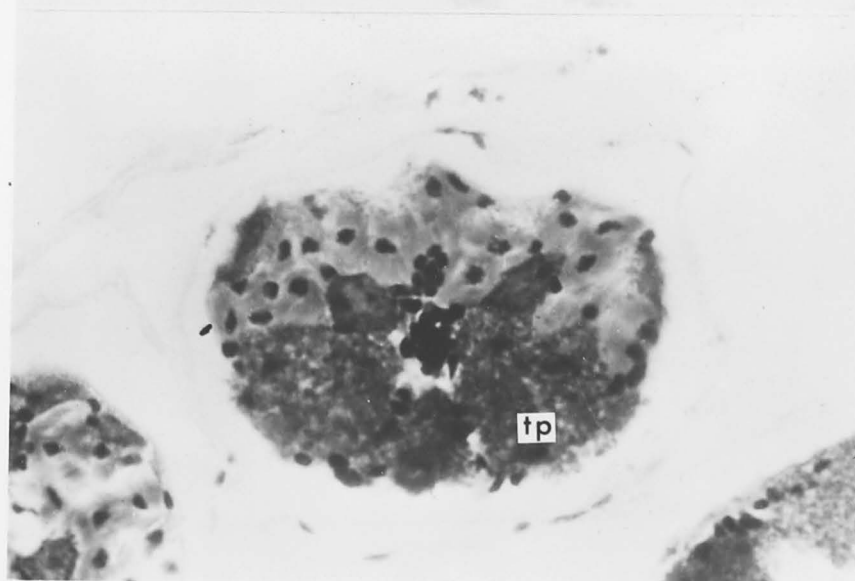
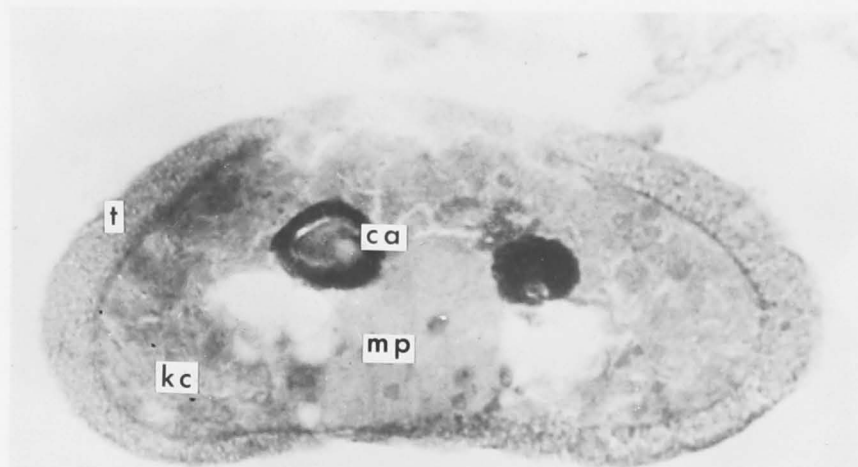


Fig. 4.5: Light micrograph. L.S. cercaria within redia.

The mucopolysaccharide cells on the dorsal surface and around the ventral sucker are PAS positive. The granular nature of the cell contents is obvious in some places (arrow). os, oral sucker; vs, ventral sucker; tp, tanned protein cells; pc, mucopolysaccharide cells; kc, keratin cells; rw, redia body wall. PAS.

Fig. 4.6: Light micrograph. T.S. cercaria within redia at level of ventral sucker (vs). Note that the peripheral mucopolysaccharide cells (pc) on the dorsal surface and around the ventral sucker are ABPAS positive and the mucoprotein cells (mp) are PAS positive. ec, excretory canal; rw, redia body wall. ABPAS.

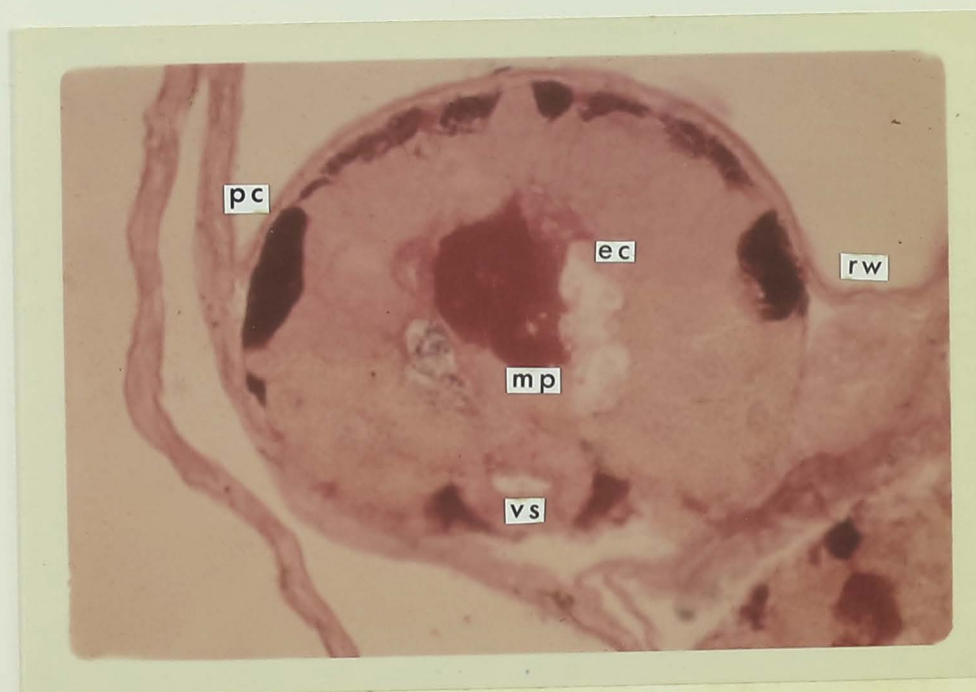
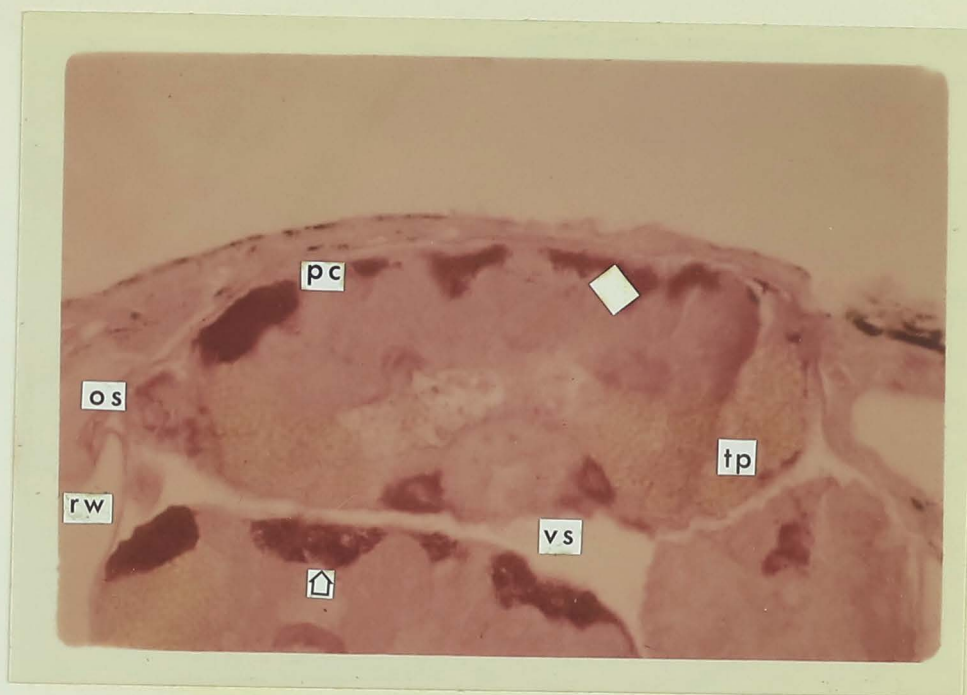
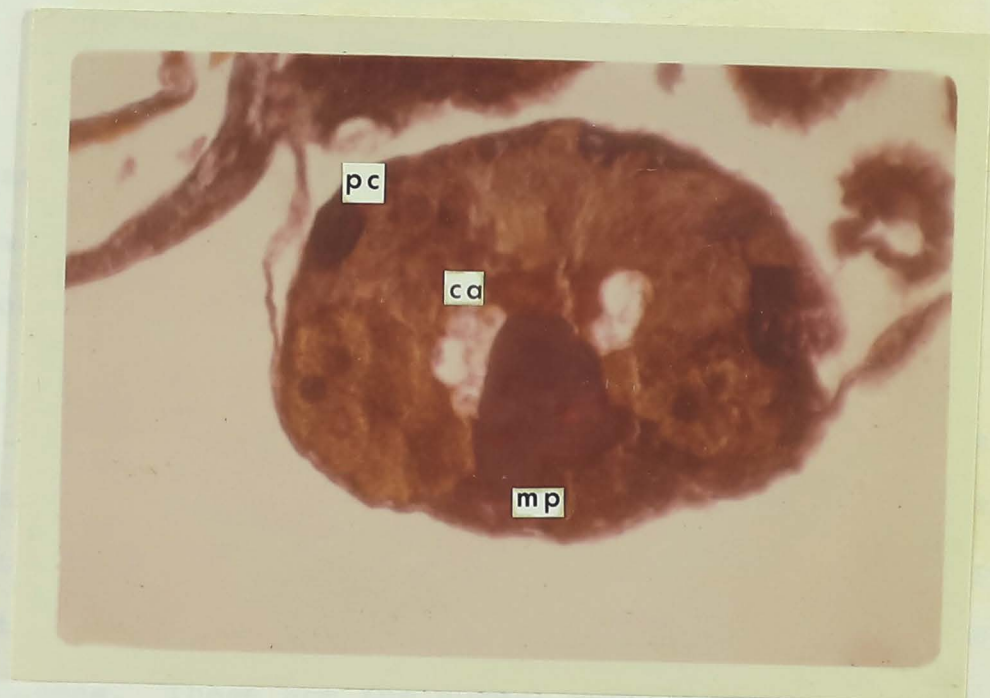


Fig. 4.7: Light micrograph. T.S. cercaria within redia approximately half way between the ventral sucker and the pharynx. The lumen of the caecum (ca) can be seen. The mucoprotein cells (mp) are close to the ventral surface. pc, mucopolysaccharide cells. Mallory's triple stain, PAS.

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used after the thioglycollic acid treatment, no staining resulted. These results indicated that both free SH groups and disulphide bonds were present in the rods.

Disulphide bonds have been demonstrated in layer IV of the inner cyst wall. The keratin cells are the only ones in which large numbers of disulphide bonds and sulphhydryl groups could be detected and thus it is assumed that the contents of these cells are used to produce layer IV.

(c) Electron microscopy

The names by which some cellular structures and organelles are known vary between different authors. The terminology used here will in general follow that used by Mercer (1961) and Kurosomi (1961).

The tanned protein cells (Figs. 4.8; 4.9)

The cells which synthesize the secretion forming the outer tanned protein layer of the cyst wall are characterised in the early stages by the possession of a well developed, particle-covered reticulum, a few mitochondria and some free ribosomes. These are the normal requirements of a proteogenic cell (Mercer, 1961) (Fig. 4.10).

The ovoid to spherical secretory granules produced by these cells have a distinctive appearance which is due to a pattern of lines and dots which show little or no electron density, and therefore in micrographs, appear white against

the black body of the granule (Fig. 4.11). The dots represent transverse sections of the lines which in favourably sectioned granules are seen to run roughly from pole to pole. In mature cells, the granules are of roughly uniform size ($2\ \mu \times 1.5\ \mu$ approximately), the reticulum is less well developed and some degeneration of the mitochondria is often evident.

In some cells, in addition to the mature granules, a separate population of much smaller granules is also present. They have the distinctive appearance of the larger granules and are also contained within smooth surfaced vesicles. The definition and clarity of the enclosing membranes is evidence that these are in fact, sections of small granules and not tangential sections of large granules. The presence of two populations of granules as indicated by size difference, suggests that there are stages of synthesis of protein and accumulation in granules which are temporally distinct (Siekevitz and Palade, 1958).

The mucopolysaccharide cells (Figs. 4.12; 4.13).

These cells are situated at the surface of the cercaria, and in the mature state after osmium fixation, can be recognized by their contents, which consist of three morphologically distinct types of granules which are all contained within smooth-surfaced vesicles.

The most numerous type of granule (Fig. 4.12, gz) is roughly oval in shape, approximately $0.75\ \mu \times 0.6\ \mu$, composed of a dense, compact, finely granular substance and has a small, eccentrically placed, less dense oval area. A second type of vesicle (Fig. 4.12, gy) is similar in size to the first but shows some variation in the texture of its contents. In some, the vesicle is filled with a fine filamentous meshwork of medium density. Granules intermediate in appearance between these and the first type of granule can be seen. This suggests that the second type of granule is an immature form of the first, and further support to this theory is given by the presence in some of the less dense granules of oval bodies similar in appearance and location to those seen in the dense granules.

The third type of granule (gx) is much smaller, of medium density, and is often separated from the dense vesicle membrane by a narrow clear space. These are very much more common in cells in the early stages of synthesis (Fig. 4.13) and may represent very early stages in granule formation.

An actively synthesizing cell contains some mitochondria, well developed and relatively numerous Golgi bodies, a rather poorly developed granular reticulum and some free ribosomes. The reticulum tends to be concentrated in

the peripheral regions of the cell and the free ribosomes in the central region, giving some degree of polarization, which is however, not as marked as in most secretory cells in mammals. In cells with numerous large granules, the reticulum is much less well developed, and the Golgi bodies and mitochondria are not as numerous.

It is likely that the pattern of granule formation is similar to that which is thought to occur in mammalian mucinogenic cells (Peterson and Leblond, 1964). The protein moiety accumulates initially in the cisternae of the particle-covered reticulum and the synthesis of the carbohydrate moiety follows in the Golgi region. Fig. 4.14 shows what could be interpreted as granule formation within a Golgi-type sac.

One of the difficulties in interpreting micrographs of this material arises from the variation in the response of the granules to fixation and staining. Polysaccharide material is notoriously difficult to stabilize (Bahr, 1954; Pearse, 1961).

The mucoprotein cells (Fig. 4.15)

The most distinctive feature of the mature cell is the appearance of the granules. After either osmium or permanganate fixation, the granules, which do not appear to be enclosed within membranes, rupture although the general

shape of the granule remains the same. This is almost certainly an artifact. Most of the granules have a thick, dense, outer edge.

Scattered throughout the whole of the cell, particle-covered elements of the reticulum can be distinguished and a few mitochondria are also present. No Golgi bodies were seen which is surprising when it is considered that the chemical composition of the granules is thought to be mucoprotein. However, the earliest stage of this cell which could be recognized was already almost full of secretory granules.

The keratin cells (Fig. 4.16)

These cells contain a relatively poorly developed particle-covered reticulum in the early stages of synthesis, but it becomes more extensive as the secretory granules develop. Occasional Golgi bodies and mitochondria are seen and there are also a number of free ribosomes.

The rod-shaped secretory granules which are always contained within smooth surfaced membranes are the most characteristic feature of these cells. The arrangement of the mature granules resembles that seen in the light microscope, although it is perhaps not quite so evident (Fig. 4.16). In longitudinal section, at low power, a single rod appears as a series of separate parallel lines spaced about 85 A apart (Fig. 4.17). At much higher magnifications

(Fig. 4.18) each of these lines can be resolved into a pair of much finer lines, each about 25 Å thick and separated by a less dense space of the same distance. There were some indications of a fairly regular series of dense particles located on each fine line, and further investigations with a microdensitometer confirmed this (Fig. 4.19). The results of scanning transversely across the lines suggest that each member of the pair of fine lines is composed of a central dense core, with two less dense areas on either side (Fig. 4.20).

In transverse section, the rods appear as tightly wound spirals averaging about 24 turns and at high magnifications, the double nature of the sheets composing the spirals is evident (Fig. 4.21).

It was concluded therefore, that the rods are formed of a spirally wound, double-layered protein sheet which if unrolled, would give a flat sheet about $30\ \mu \times 5\ \mu$.

In the centre of the scroll, a dense granular substance forming a 'core' was seen. The nature and function of this substance is unknown.

In immature cercariae, cells containing scrolls in all stages of development were seen. In the earliest stage noted, the beginnings of scrolls were seen as short, curved sheets, in some cases not even forming the first turn of the

spiral (Fig. 4.22). As development proceeds the sheets lengthen, and simultaneously the rolling up process takes place (Fig. 4.23), so that scrolls with any intermediate number of turns up to the maximum number of the mature rod have been seen. There is a remarkable synchrony in development of the scrolls within any one cell, as evidenced by the small variation in the number of turns.

It seems likely that synthesis and accumulation of the precursor amino acids and polypeptides takes place on ribosomes attached to the surface of the reticulum, and the sheets form in the sacs. The process of formation of the double protein sheet would seem to have its closest analogy in fibre formation, which can be considered as a particular macromolecular association of previously formed protein molecules. A major difference however, is that scroll construction occurs within individual vesicles and rolling-up occurs at the same time. Even initially, the vesicles must have the form of a long cylinder. The lack of rigidity of the membrane makes it unlikely that it can act as a mould since, in some cases, the free end of the spiral distorts the membrane considerably (Fig. 4.23, arrows). The formation of the scroll probably results from a spontaneous aggregation of the precursor, a process which may have some affinities to crystallization.

Fig. 4.8: Electron micrograph. Tanned protein secretory cell containing two populations of granules as judged by their size (tg, arrows). There is a well developed, particle-covered reticulum (r), some free ribosomes (rb) and a degenerate mitochondrion (m). The granules show the characteristic fine structure of lines and dots and are enclosed within vesicles (vm, vesicle membrane) which in this case have become broken in places due probably to damage during dehydration. Osmium tetroxide, Millonig's lead.

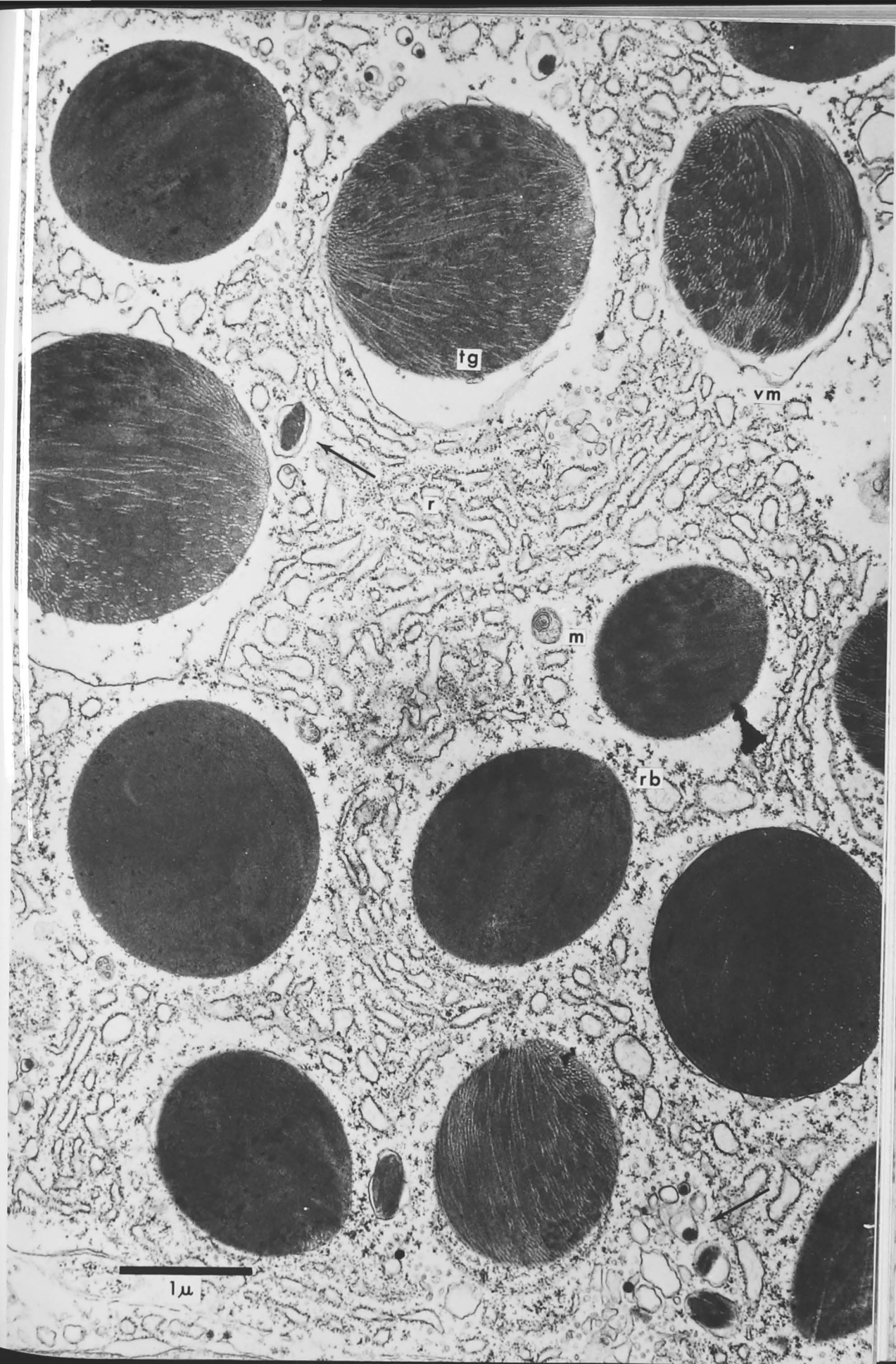


Fig. 4.9: Electron micrograph. Tanned protein secretory cell containing granules of slightly different although basically similar appearance to those in Fig. 4.8. A multivesicular body (mv) and a degenerate mitochondrion (arrow) are obvious. vm, vesicle membrane; m, mitochondrion; r, reticulum; rb, ribosomes. Osmium tetroxide, Millonig's lead.

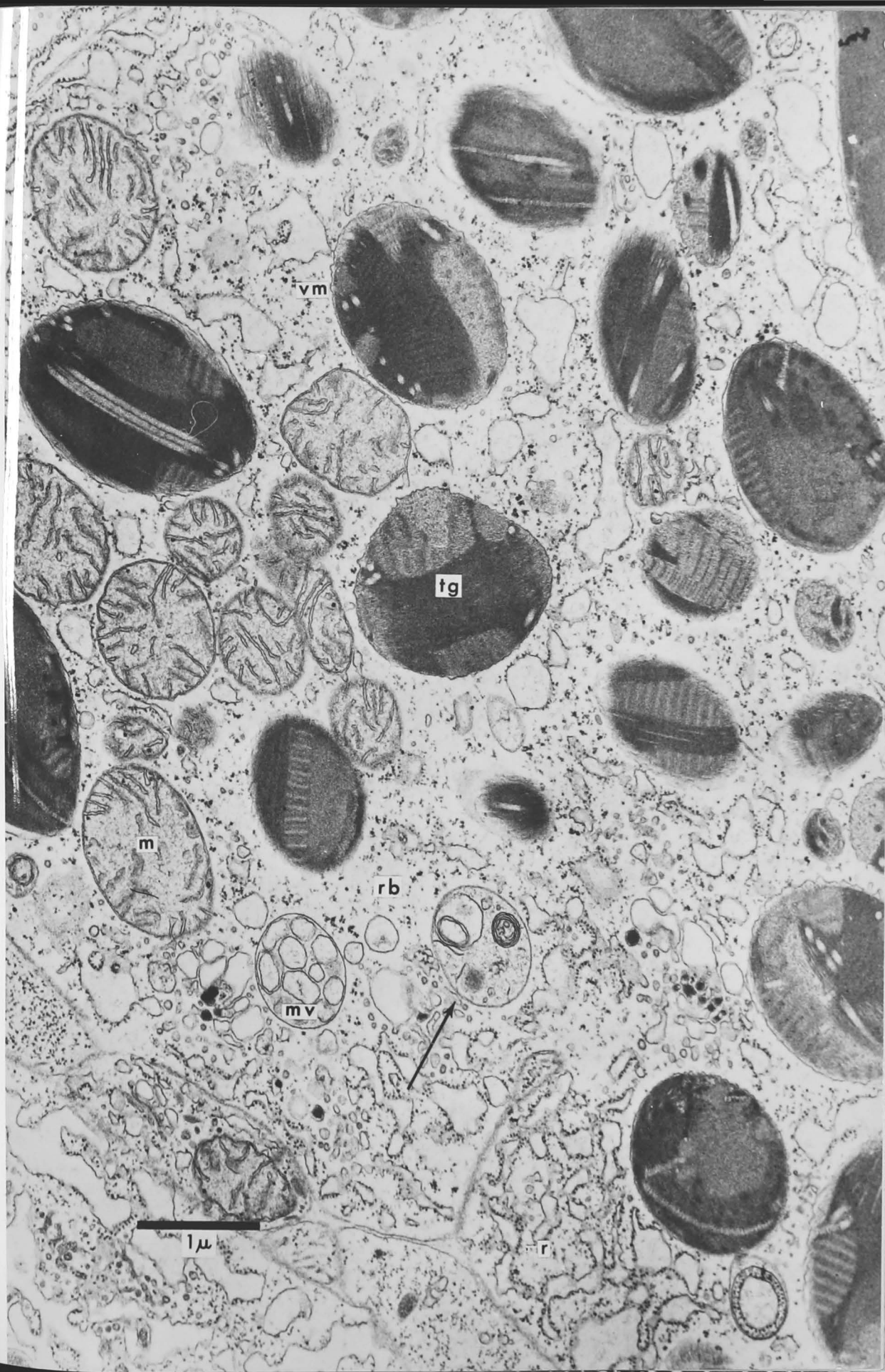
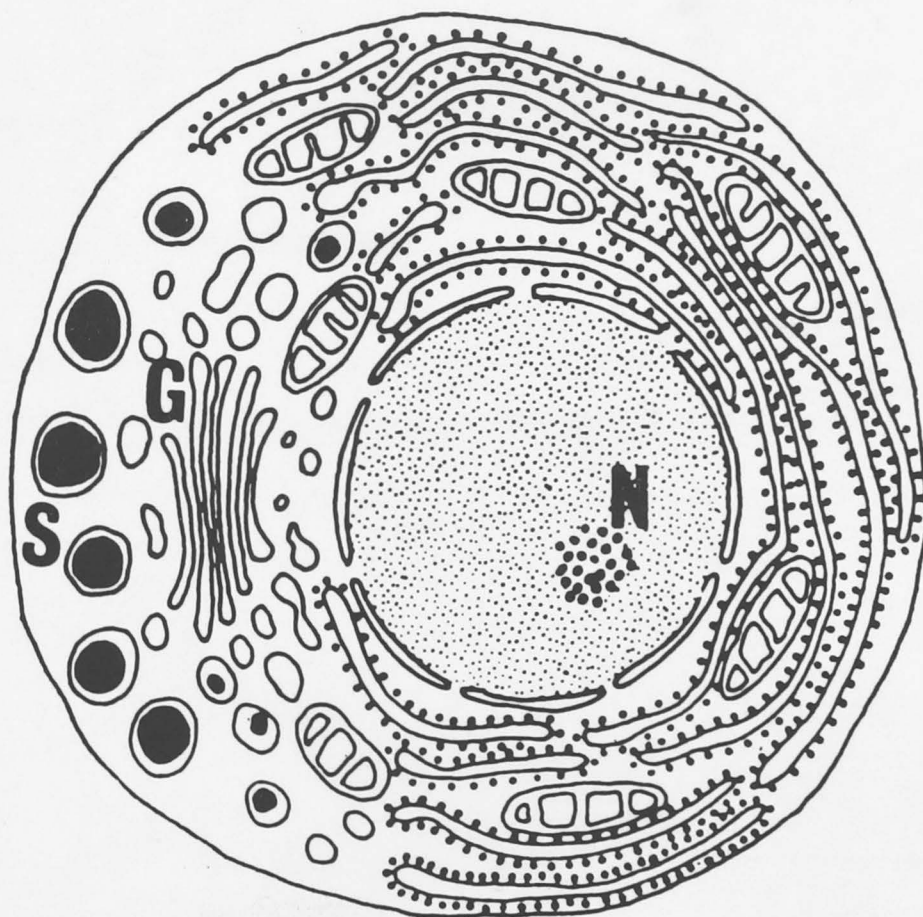


Fig. 4.10: Principal cytological features of a cell synthesizing protein for secretion. N, nucleus; G, Golgi body; S, secretory granule. The particle-covered reticulum is well developed. (Used with the kind permission of Dr E.H. Mercer).



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Fig. 4.11: Electron micrograph. High magnification view of a tanned protein granule contained within a vesicle membrane (vm). The channels (arrow) run across the granule. Osmium tetroxide, Millonig's lead.

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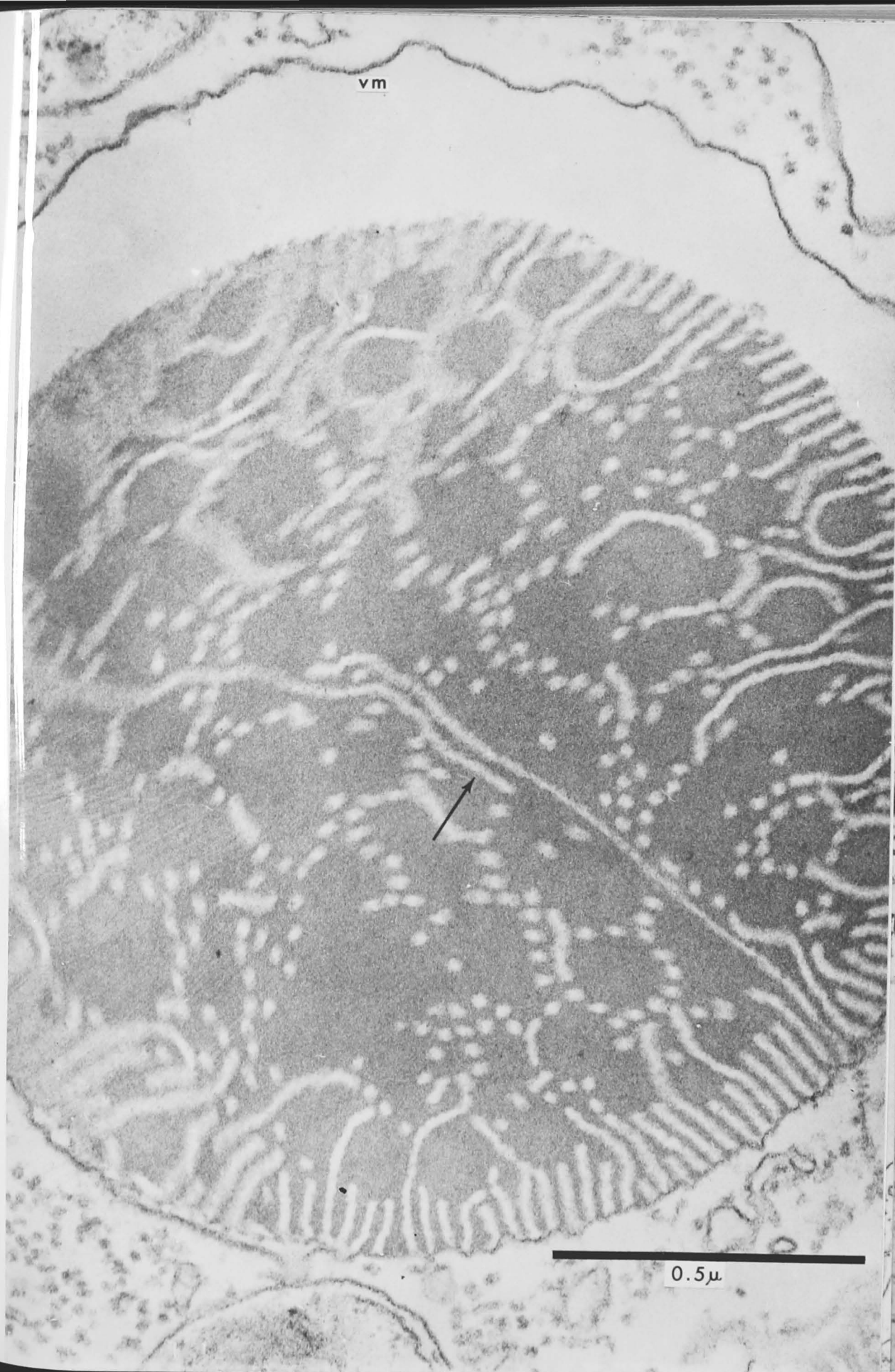


Fig. 4.12: Electron micrograph. Mature peripheral mucopolysaccharide cell. The three types of granules (gx, gy, gz) may represent stages in formation, gz being the most advanced. The less dense ovoidal area at one pole of these granules is obvious (arrow). ks, keratin scroll; pm, plasma membrane. Osmium tetroxide, Millonig's lead.

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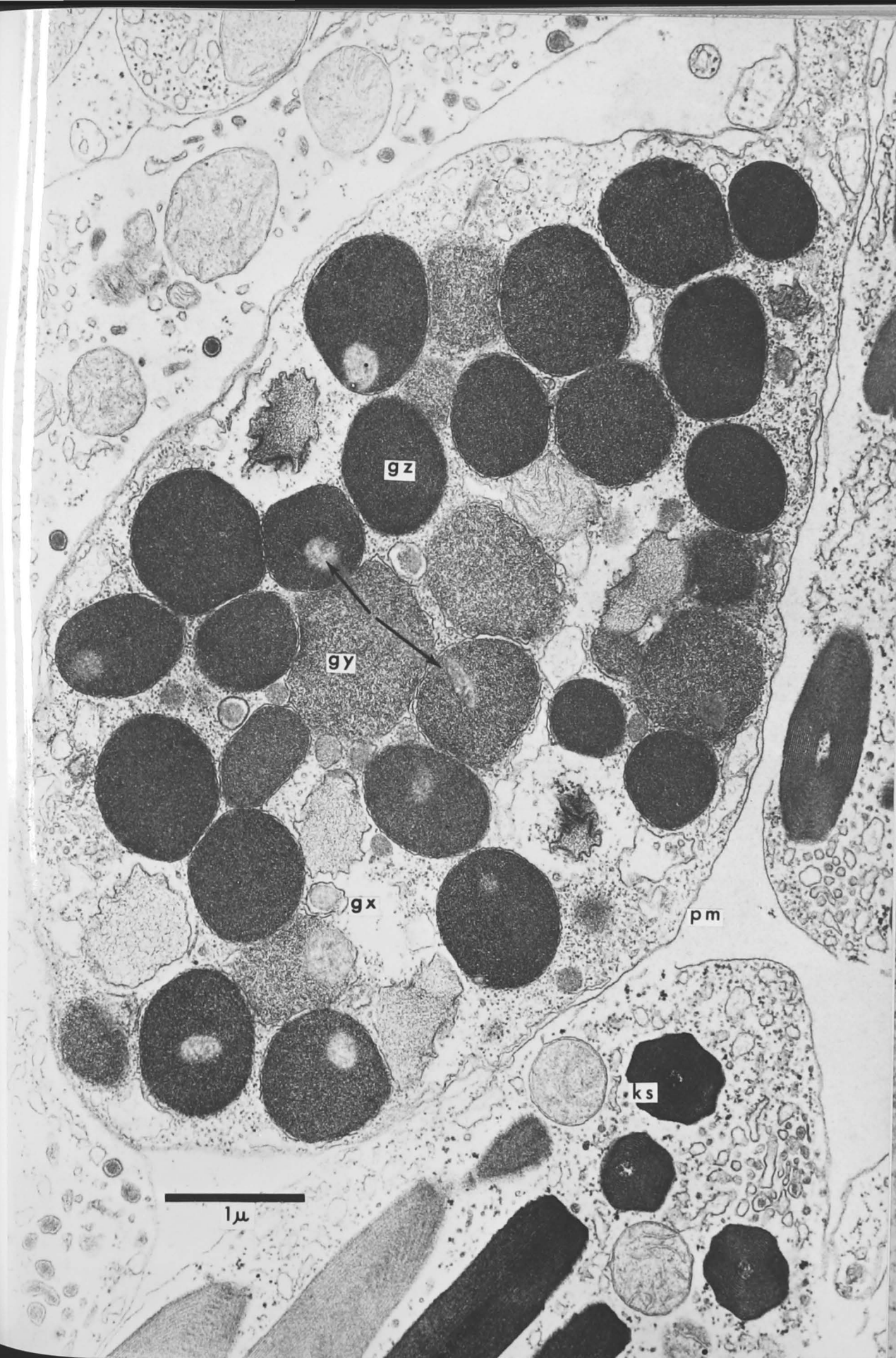


Fig. 4.13: Electron micrograph. Immature peripheral mucopolysaccharide cell containing three types of granules (gx, gy, gz) which are similar to those of the mature cell (Fig. 4.12). There are three prominent Golgi bodies (gb) all of which show some indications that they may be involved in formation of the granules. The reticulum (r) is concentrated mainly at the periphery of the cell. ee, embryonic epithelium; mc, muscles; m, mitochondrion; pm, plasma membrane; n, nucleus. Osmium tetroxide, Millonig's lead.

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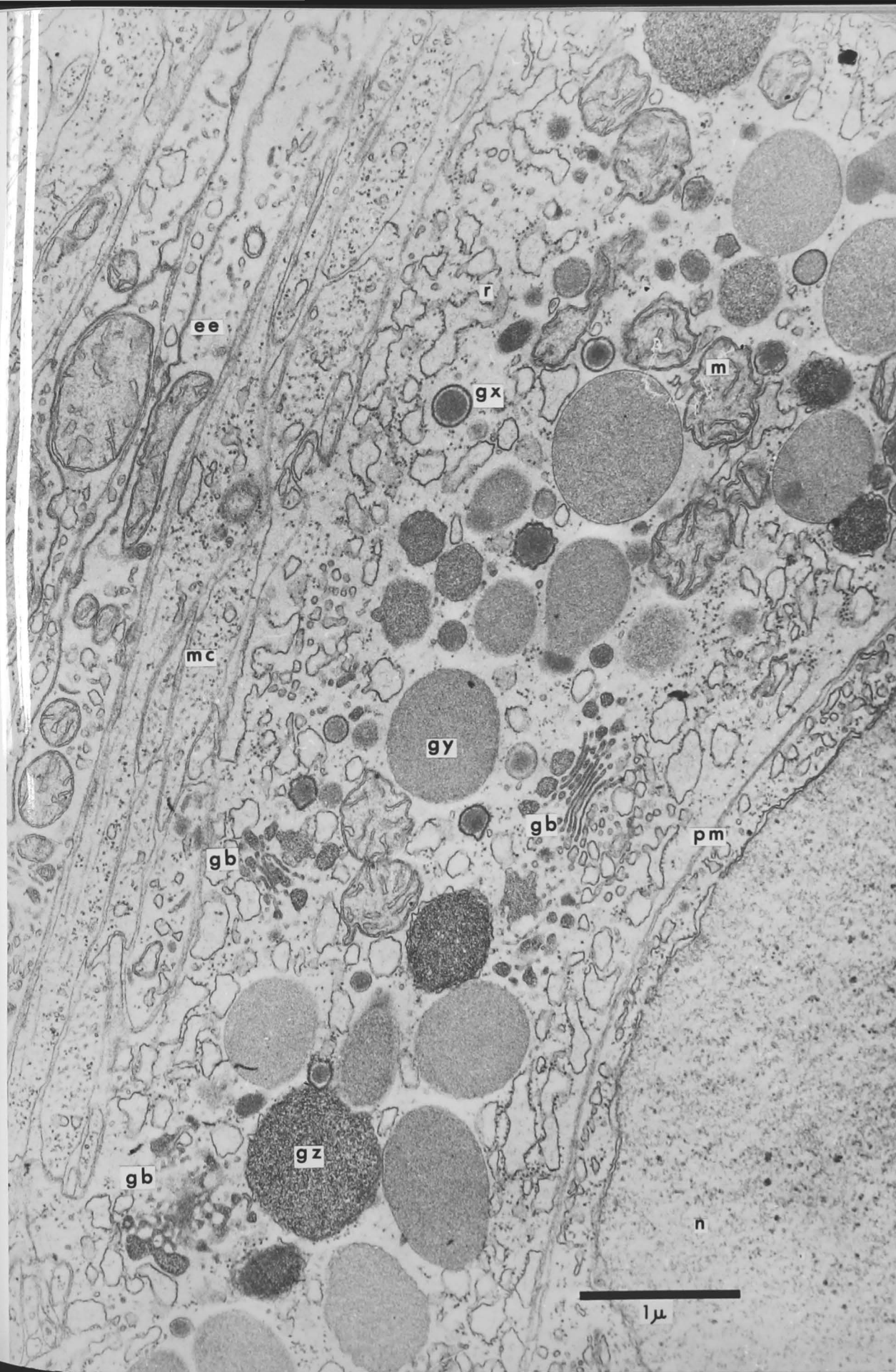


Fig. 4.14: Electron micrograph. The Golgi body (gb) surrounds a granule (g) and another similar granule is in the cytoplasm nearby. r, reticulum; pm, plasma membrane. Osmium tetroxide, Millonig's lead.

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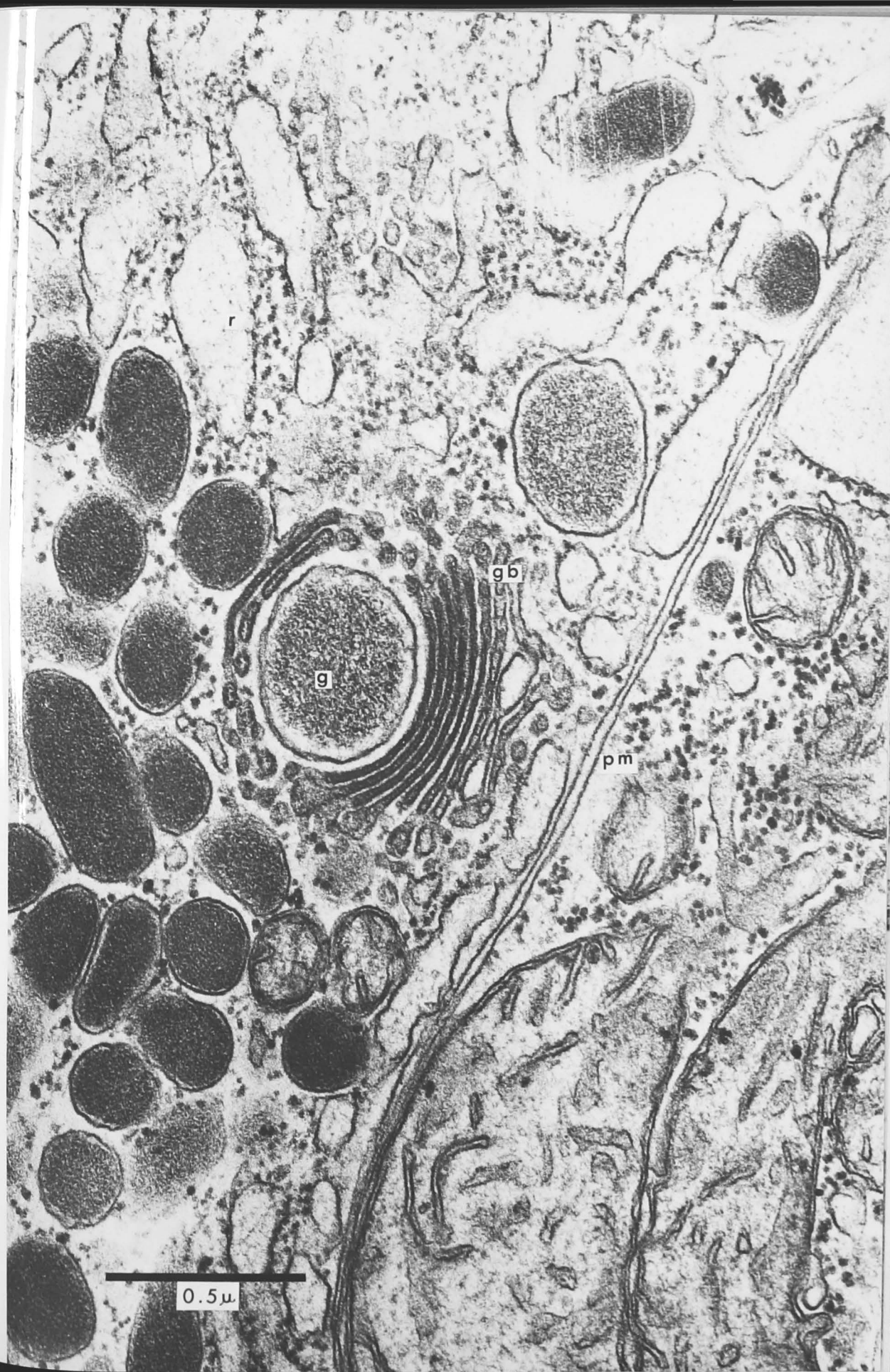


Fig. 4.15: Electron micrograph. Mucoprotein cell containing granules (g) which have a dense outer edge and are not contained within membranes. Most of the granules have ruptured (arrow). m, mitochondrion; r, reticulum. Osmium tetroxide, Millonig's lead.

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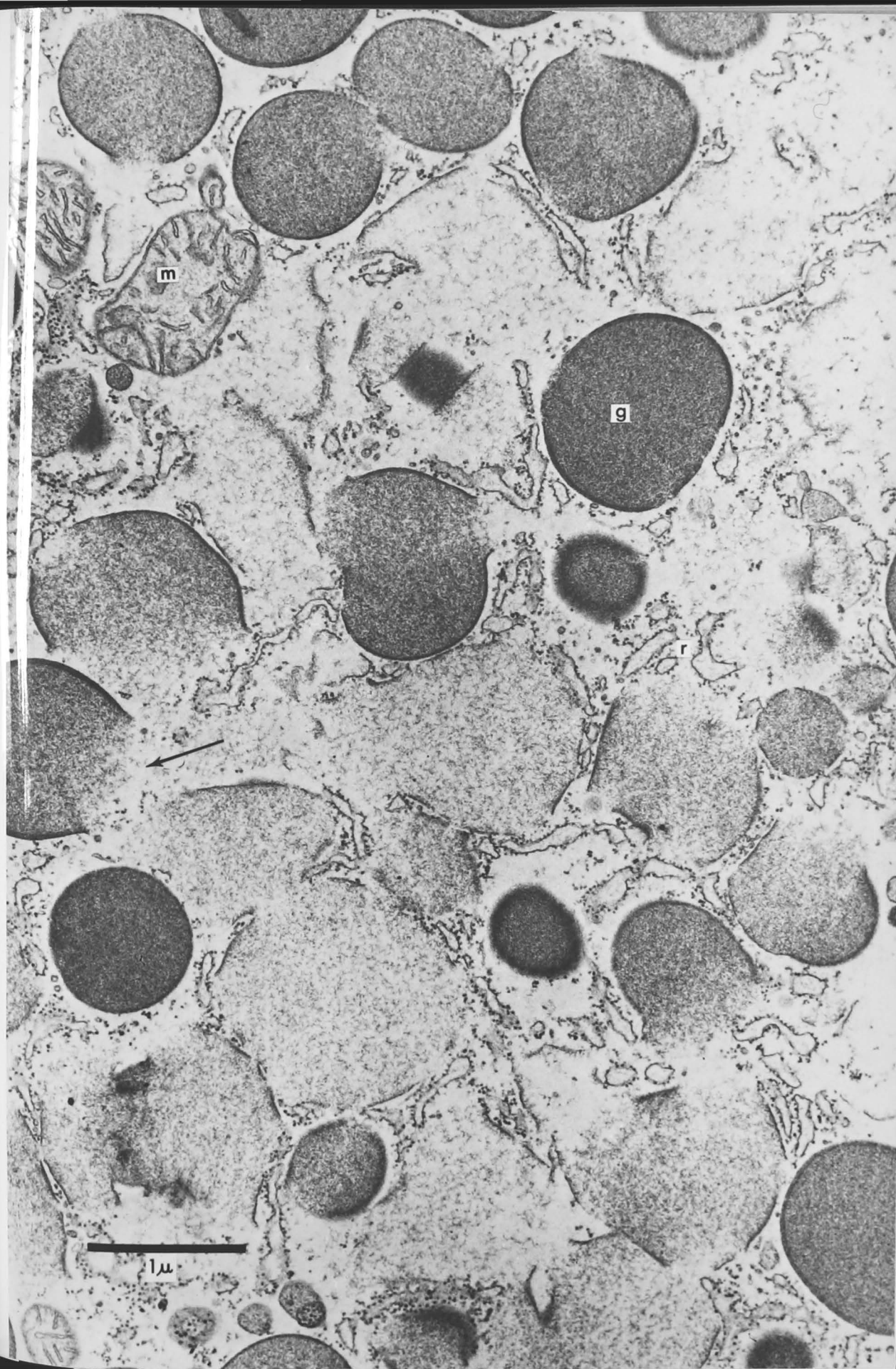


Fig. 4.16: Electron micrograph. Low power survey picture to show the arrangement of parallel keratin scrolls (ks) in bundles. The scrolls can be seen in longitudinal and in transverse section. The embryonic epithelium (ee) has become filled with tanned protein granules (tg) and carbohydrate-protein granules (g). mc, muscles; pc, peripheral mucopolysaccharide cell; n, nucleus; pm, plasma membrane. Potassium permanganate, Millonig's lead.

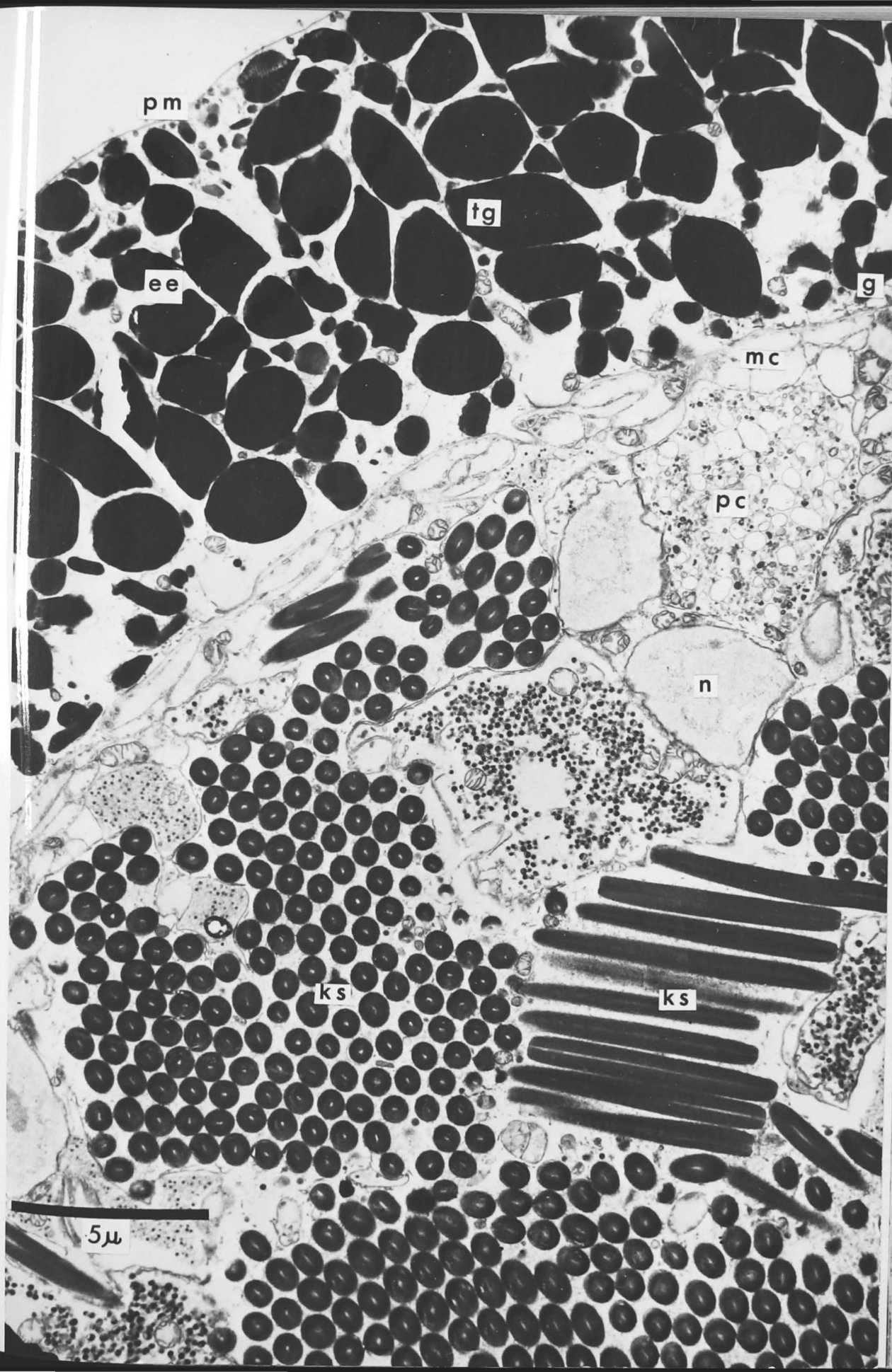
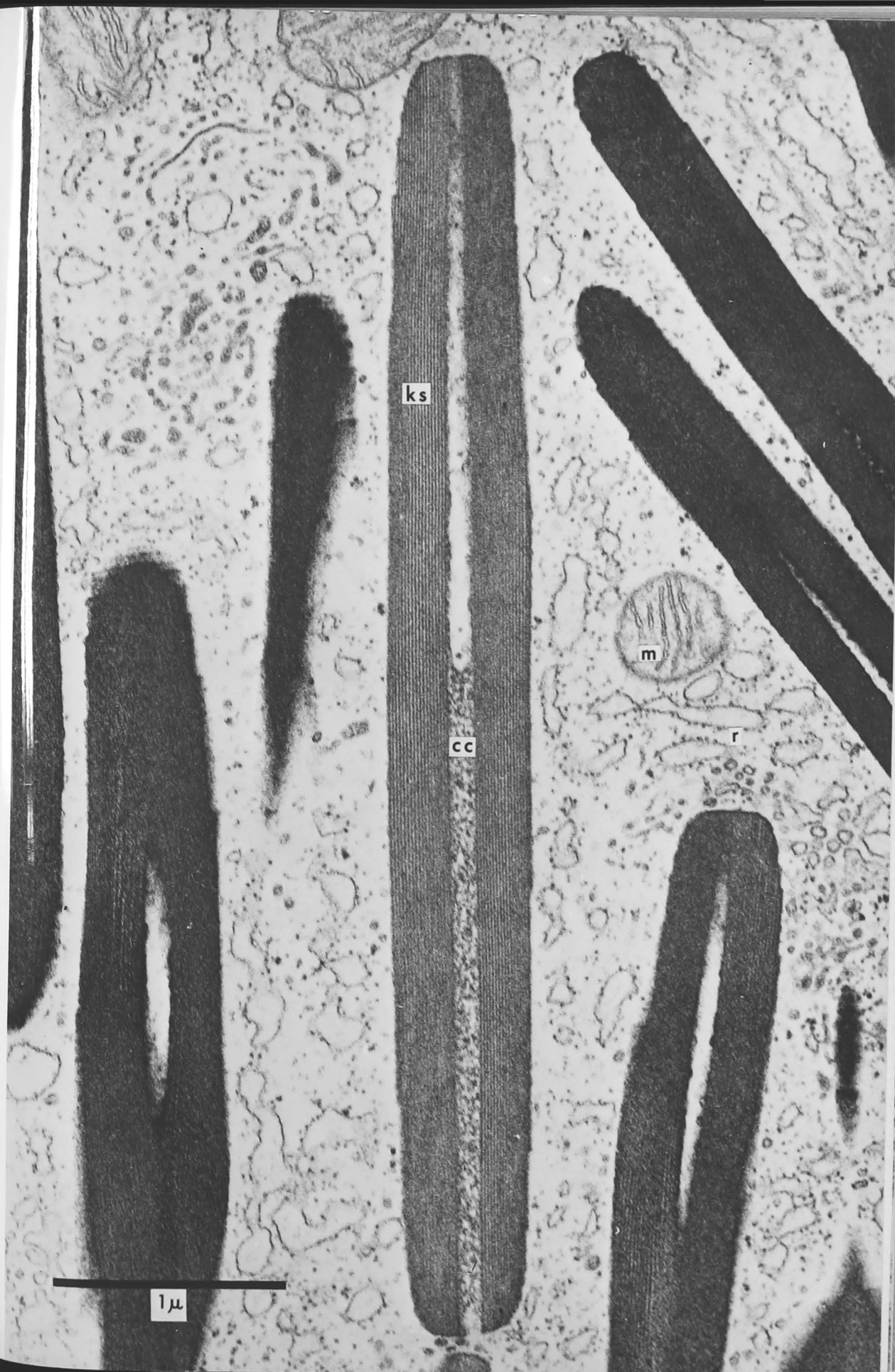


Fig. 4.17: Electron micrograph. Longitudinal section through a keratin scroll (ks). The central core (cc) is prominent at one end. m, mitochondrion; r, reticulum. Osmium tetroxide, Millonig's lead.



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Fig. 4.18: Electron micrograph. High magnification view of portion of a keratin rod showing the arrangement of double parallel lines. The central core (cc) of amorphous material is present. Osmium tetroxide, Millonig's lead.

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Fig. 4.19: Microdensitometer trace produced by scanning section of keratin scroll longitudinally. The trace indicates the presence of a fairly regular series of denser regions arranged along the 'lines' of the scroll.

Fig. 4.20: Microdensitometer trace produced by scanning section of keratin scroll transversely. The trace indicates that each 'line' of the scroll has a dense core with a less dense edge on either side.

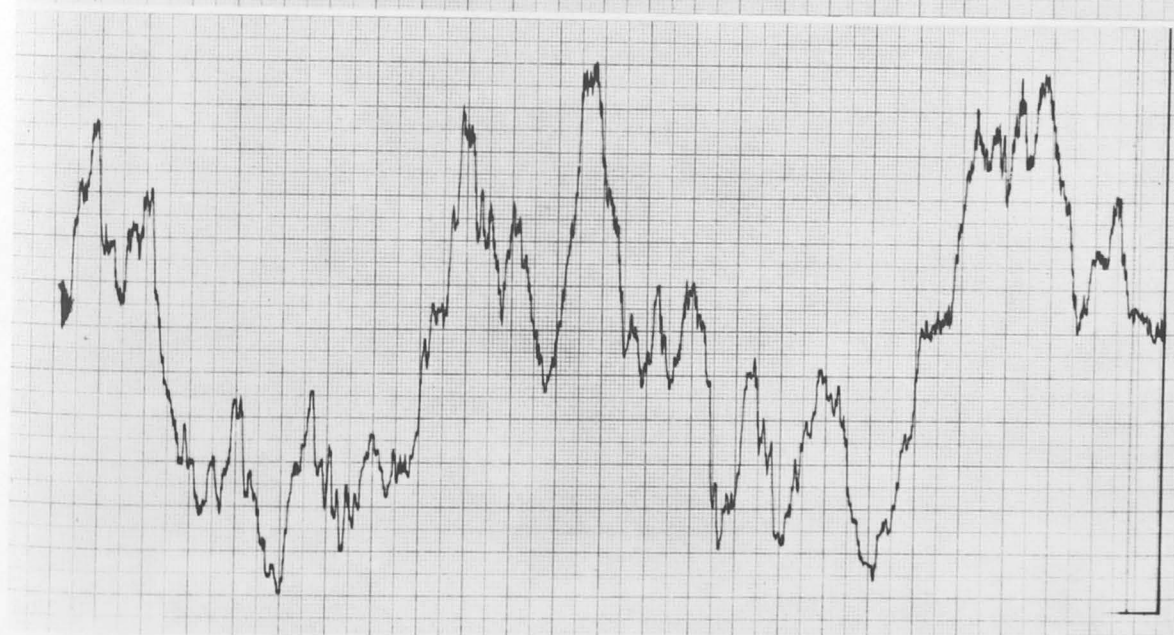
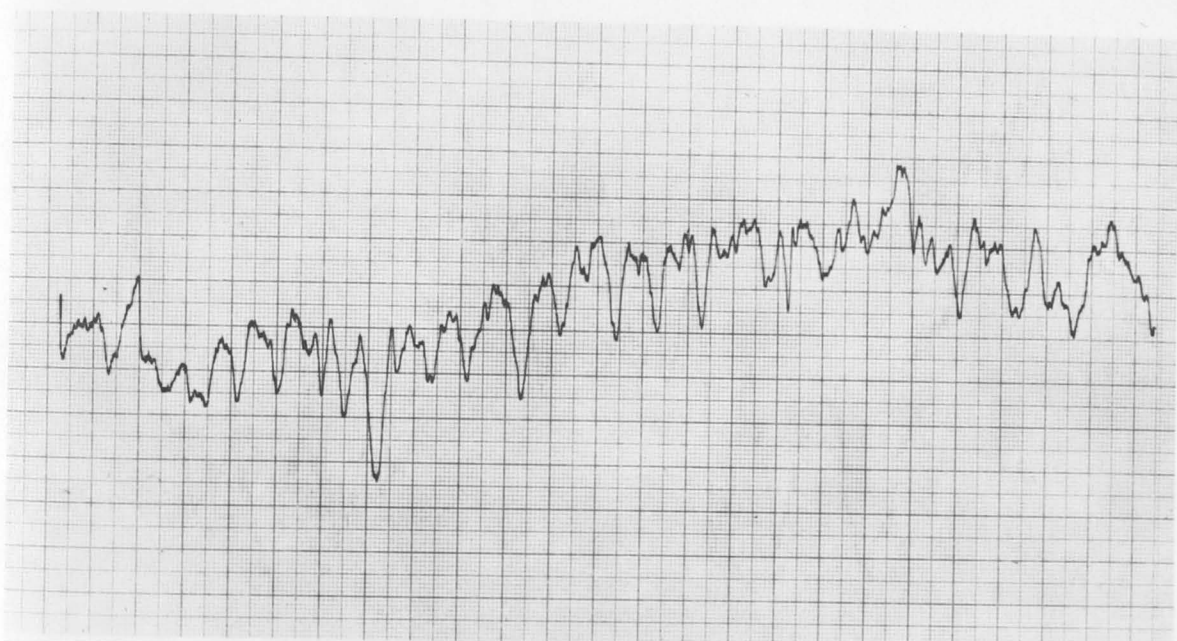


Fig. 4.21: Electron micrograph. Transverse section of a keratin scroll (ks) which is not quite fully formed. There are 20 turns and the double nature of the spiral is obvious. Some indications that there is a regular series of granules along the protein sheet can be obtained. Note the material in the core of the scrolls at the bottom of the micrograph. The origin and function of this material is unknown. m, mitochondrion; cr, crista; r, reticulum; rb, ribosomes. Osmium tetroxide, Millonig's lead.

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Fig. 4.22: Electron micrograph. A very early stage in formation of the keratin scrolls which have not yet completed one turn (arrow) but are enclosed within vesicles (vm). r, reticulum; gb, Golgi body; n, nucleus. Osmium tetroxide, Millonig's lead.

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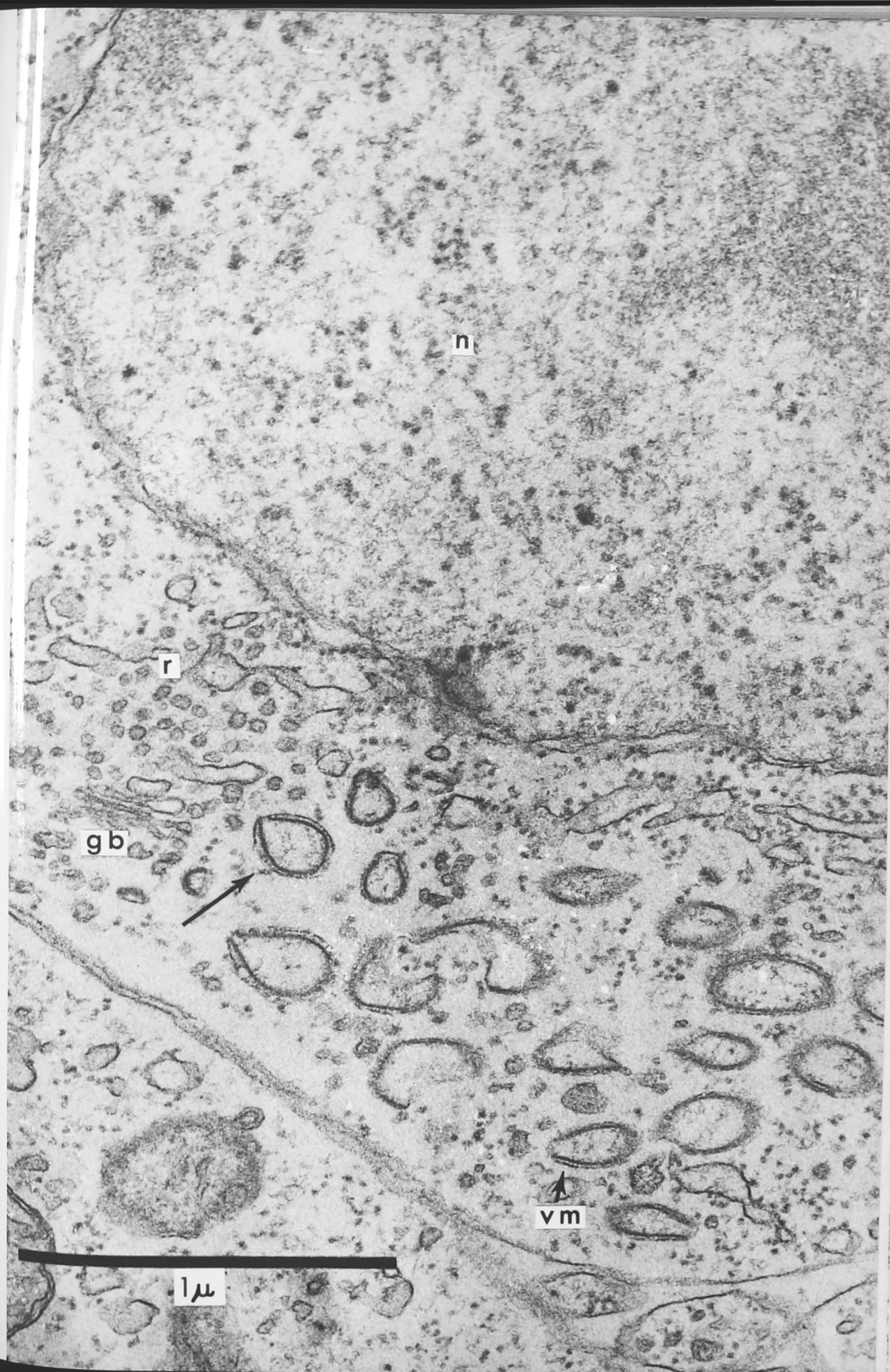
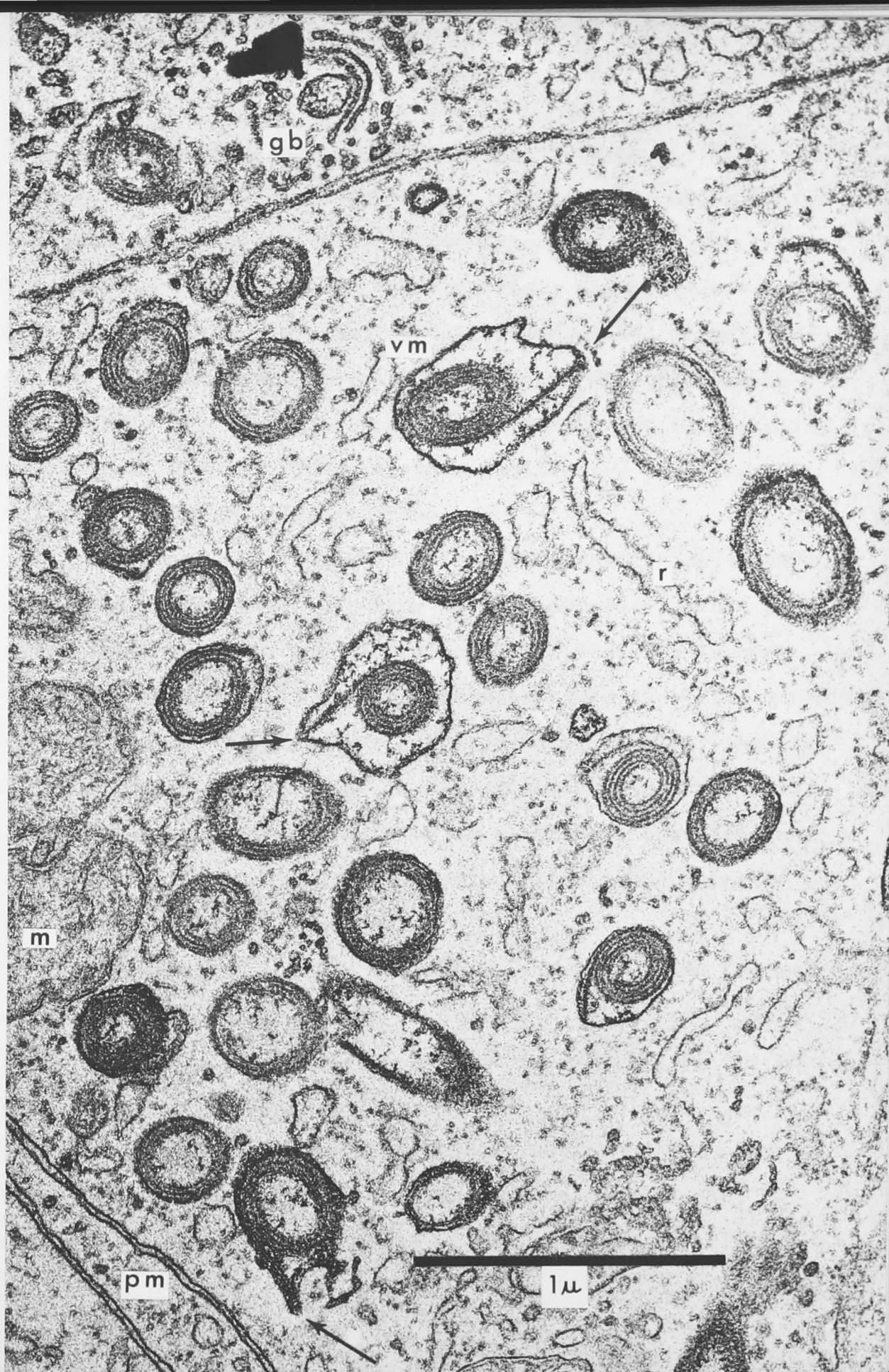


Fig. 4.23: Electron micrograph. A later stage in the development of the keratin scrolls where some lengthening and rolling-up of the sheet has taken place. In some cases the vesicle membrane (vm) is distorted by the free end of the scroll (arrows). gb, Golgi body; m, mitochondrion; r, reticulum; pm, plasma membrane. Osmium tetroxide, Millonig's lead.



2. The Behaviour of the Cercaria during Encystment (Fig. 4.24)

After escaping from the snail, the cercaria swims actively, tending to remain in the upper layers of the water. When it comes in contact with a solid surface, either sucker may become temporarily attached and contractions and elongations of the body also take place. The cercaria may resume swimming and later, on contacting the surface again, repeat this procedure. Finally, however, this exploratory phase is followed by the attachment phase.

Attachment takes place very rapidly, lasting about 30 sec., and involves a flattening of the cercaria against the substrate so that it occupies about twice the area it previously did (Fig. 4.24 d). At the same time, the tail straightens and appears completely rigid. In this position, the tail lies approximately parallel to the substrate and then, while it remains rigid, its distal end is slowly elevated at a uniform rate until the tail is approximately perpendicular to the substrate (Fig. 4. 24 e).

The contraction phase follows immediately, when the body of the cercaria rounds up as it contracts away from an outer layer which remains behind because it is attached to the substrate (Fig. 4.24 f).

The animal should now be termed a metacercaria. Contraction proceeds until the metacercaria occupies about two-thirds of the area enclosed by the outer cyst wall. At

about the time the contraction is at a maximum, the tail, which up to this time has remained rigid, resumes beating in a manner which is basically similar to that used during swimming, but which now seems less well coordinated.

When the metacercaria has contracted fully, it commences a complex series of vigorous movements within the forming cyst wall and these continue for about 1 hr. There are three distinct kinds of movement employed by the metacercaria at different times but there are also transitional types of movement (Figs. 4.24 h-k).

Initially, the movements of the metacercaria are made with the centre of the body fixed, presumably by the ventral sucker. The two halves of the body move independently of each other so that the extreme anterior and posterior ends describe short arcs. This tends to be a very jerky movement. After some time, which varies between metacercariae, the second type of movement is superimposed over a modification of the first. During this phase the central part of the body is no longer permanently fixed but the animal now is able to rotate within the cyst wall and at the same time maintain the independent movements of the anterior and posterior ends. The direction of rotation is not constant. The metacercaria may rotate completely up to three or rarely, four times in a

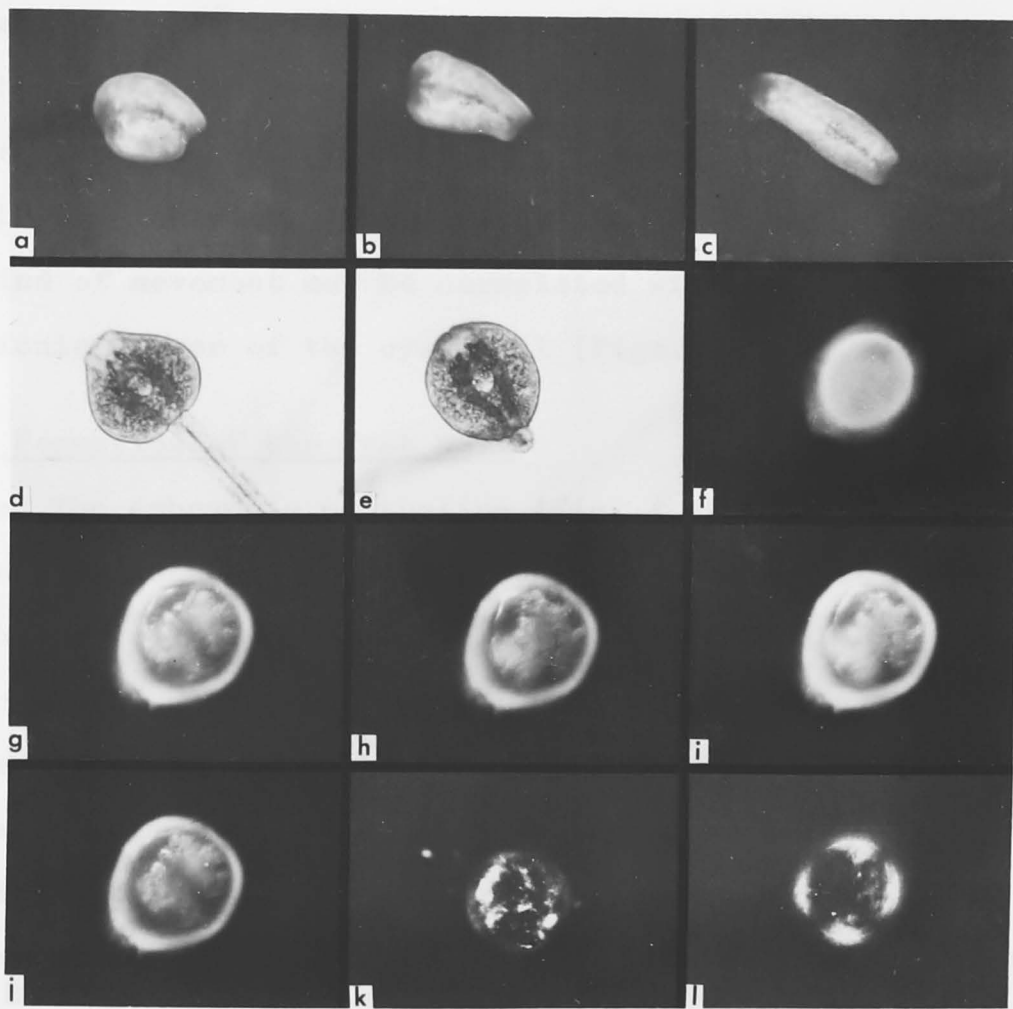
clockwise direction and then reverse its movements and rotate in a counter-clockwise direction.

The third type of movement follows and in this phase, while some rotation persists, the body contracts continually so that the surface has a continuous flowing motion in all directions.

A reasonable interpretation of the behaviour of the cercaria is that the attachment phase is initiated by a tactile stimulus, possibly from the ventral region and more particularly the ventral sucker, which leads to a firm attachment by both suckers. The tango-receptors near the oral sucker which were described by Dixon and Mercer (1965a) could also play an important part. The transverse muscles then contract vigorously at the same time as the longitudinal and circular muscles are relaxed, resulting in flattening of the body of the cercaria against the substrate. The animal, by contraction of the circular and longitudinal muscles, then rounds up and so contracts away from an outer layer which continues to enclose the cercaria, and which forms what is in effect, the outer cyst wall. At the same time the tail becomes rigid, indicating that its attachment to the body has broken. The proximal end however, remains held down on the substrate by the outer cyst wall, and if it were not for this attachment, the tail would float away. As it is, the distal

Fig. 4.24: Series of light micrographs taken from a 16 mm cine film of an encysting cercaria.

- a-c, the exploratory phase showing the extensions and contractions of the body. Dark field.
- d, attachment, in which the body of the cercaria is flattened against the substrate and the tail straightens. Bright field.
- e, the cercaria is still flattened against the substrate and the tail has 'floated' to a vertical position. Bright field.
- f, the contraction phase in which the metacercaria rounds up within the detached outer cyst wall which adheres to the substrate. Dark field.
- g-k, the movements of the metacercaria within the cyst wall. It is not possible to distinguish between the various sorts of movement from single frames. Dark field.
- i, the appearance of the developing cyst under polarized light before the formation of the keratinized Layer IV. The birefringence is restricted to the keratin scrolls still within the metacercaria.
- j, after the formation of the keratinized layer, very little birefringent material remains within the metacercaria and the cyst wall shows the 'Maltese cross' effect. Polarized light.



end of the tail 'floats' upwards so that in the final position, the tail is perpendicular to the substrate. Since the nerves innervating the tail are now broken, its subsequent movements must be due to endogenous contractions of the muscles of the tail.

The movements of the metacercaria within the cyst wall are probably connected with the secretion of the cell contents and with the moulding of the cyst wall. It seems likely that each kind of movement may be correlated with the formation of a particular layer of the cyst wall (Figs. 4.24 l, m).

3. The Formation of the Cyst Wall

(a) The embryonic epithelium (Fig. 4.25)

On examination in the electron microscope of sections through cercariae in all stages of development, a thin outer layer could be distinguished, which is bounded externally by a plasma membrane and contains a few small mitochondria and some small granules. Very infrequently, a nucleus was also seen within this layer and it became obvious that the layer is composed of a small number of thin, flattened cells which completely cover the surface of the cercaria, and is analogous to an epithelium. To denote its early origin in this larval form, it will be referred to as an embryonic epithelium. It is of interest to note that the embryonic epithelium

also invests the tail, and it is probable that, when the cells of the tail are budded off, they push out from the body of the cercaria and carry the embryonic epithelium with them. The number of cells which form the epithelium could not be determined but, taking into account the rarity of nuclei, it is evident that there are only a few cells.

The embryonic epithelium rests on a basement membrane, which does not appear to have any fibrous components, and the circular and longitudinal muscle cells lie immediately beneath this.

The function of the embryonic epithelium other than in cyst formation (see later) is obscure. It probably plays some part in absorption of food material by pinocytosis while the cercaria is within the redia (Fig. 4.28).

It is interesting to note that a previously undescribed protoplasmic sheet from the redia body wall is attached to the outer plasma membrane of the embryonic epithelium (Fig. 4.26). This may function in maintaining the developing cercaria in position although the advantage of this appears doubtful.

(b) The outer cyst wall

After staining paraffin sections of cercariae within rediae with aqueous BPB, the granules of the tanned protein cells stain a brilliant blue colour (Fig. 4.4). In some

cercariae still within the rediae, and in all cercariae free in the snail tissue, these granules form a thick layer which almost completely envelops the body of the cercaria and which is limited externally by a very thin membrane which is not visible in all places. No BPB positive material remains within the original cells (Fig. 4.27). In some cases the granules stained as brilliantly as those still in the cells of the cercariae nearby, but in other cases the colour produced was much less intense. When the diazo reaction for phenols was used on whole mounts of free swimming cercariae (in which the tanned protein granules had been secreted), a less intense colour resulted than when this reagent was used on cercariae with the tanned protein cells intact. These observations suggest that the tanning process occurs after transfer of the granules into the embryonic epithelium.

Granules giving a positive diastase-fast PAS reaction were also noted forming a thin layer over the surface of the cercariae, even while the tanned protein cells were still intact. In sections of free-swimming cercariae, the PAS positive granules form a layer near the inner edge of the embryonic epithelium and the tanned protein granules are concentrated towards the outer edge.

The observations with the electron microscope confirmed and amplified the results obtained with the light microscope.

In immature cercariae developing within the redia, the cells of the embryonic epithelium sometimes contain a few granules which appear similar to those of the peripheral mucopolysaccharide cells (Fig. 4.28). Some of the granules show some similarity to the granules of the central mucoprotein cells, in that they are not enclosed within a membrane and the outer edge of the granule is denser. Before the cercaria leaves the redia however, the characteristic granules of the tanned protein cells are transferred into the embryonic epithelium, and the amount of material is so great that the embryonic epithelium becomes grossly distended (Fig. 4.29). The histochemical evidence suggests that the tanning process takes place after transfer of the granules, although no changes in fine structure were evident. Tanning involves essentially the formation of cross linkages between protein molecules and need not cause any structural changes other than at a molecular level.

After transfer of the tanned protein granules to the embryonic epithelium, the granules of carbohydrate-protein material tend to congregate near the inner surface of the embryonic epithelium, so that some stratification of the two sorts of granules becomes apparent (Fig. 4.29).

The contents of the tanned protein cells, including mitochondria and secretory granules, pass into the embryonic

epithelium through channels formed by invaginations of the basement membrane between adjacent muscle cells (Fig. 4.30). The way in which the mucopolysaccharide granules of the peripheral cells reach the embryonic epithelium is not known, but it probably involves transfer of only relatively few of the granules to the embryonic epithelium. The mucoprotein granules are probably secreted through channels between the muscle cells in a similar manner to the tanned protein granules (Fig. 4.31).

When the embryonic epithelium is shed, the cigar-shaped particles of each tanned protein granule appear to slip relative to each other, with the less dense channels possibly acting as 'fracture lines'. The partly dissociated granules then adhere to each other to form the loose meshwork which is characteristic of the formed cyst wall (Figs. 3.1; 3.2).

The initial rotatory movements of the metacercaria presumably serve to draw out the carbohydrate-protein granules into poorly defined fibres (Figs. 3.1; 4.32).

(c) The inner cyst wall

Layer III: The details of formation of the three components of layer III were not fully elucidated. This was mainly due to the difficulty in characterising the granules, which in turn was due to their variable appearance, even after similar treatment. From the histochemical data, it seems reasonable to

conclude that the contents of the central mucoprotein cells are used to form layer IIIa and that the contents of the peripheral mucopolysaccharide cells form the layers IIIb and IIIc.

Sections of partly formed cysts fixed 10 min. after encystment commenced (Fig. 4.32), showed that between the outer surface of the cercaria and the now fibrous-textured layer II, there is a jumbled collection of granules of two major types: (a) dense granules of variable shape similar to those seen in the mucopolysaccharide cells and still contained within a membrane and (b) less dense granules, also of variable shape, but lacking an enclosing membrane; the most distinctive feature of the latter granules is their zigzag pattern, formed from a large number of thin, parallel, dense, transverse bands. The origin of these granules is not known as they do not resemble any intracellular granules in the cercaria. It seems possible, however, that these may be the granules of the mucoprotein cells which have undergone a structural change after secretion. The absence of an enclosing membrane is one common feature.

Two plasma membranes enclose this aggregation of granules. One lies next to the basement membrane overlying the muscle cells, and the other, which is not always visible,

lies just inside the fibrous elements of layer II. The origin of these plasma membranes is unknown.

At a slightly later stage of formation (Fig. 4.33), the layer IIIa has formed although some condensation and consolidation is still to take place. The layers IIIb and c are in the process of formation and appear at this stage as a finely granular matrix containing many mitochondria and remnants of reticulum.

Plasma membranes also enclose this layer, one below layer IIIa and the other just above the outer basement membrane. The origin of these is also unknown.

When the formation of layer IV has begun (Fig. 4.34), layer III has a similar appearance to that in the fully formed cyst wall although it is less densely packed. The plasma membranes which separated the various sublayers in earlier stages are no longer visible. Layer IIIa appears as a dense, relatively loose, granular layer, still containing a few small, degenerate, empty vesicles. Layers IIIb and c, which are not separable in the electron microscope (see Chapter 2), appear as a single homogeneous layer of medium density.

The peripheral mucopolysaccharide cells utilise a holocrine-type secretory process in which almost the complete contents of the cells are extruded, presumably through the

channels between the muscle cells. The nucleus and a few of the cellular organelles remain in the cell (Fig. 4.35). Little is known about the method of secretion of the contents of the mucoprotein cells (Fig. 4.31), except that it probably also occurs through channels between the muscle cells.

Layer IV: In cercariae within the snail, the cells containing rods are concentrated in the dorsal half of the body. In the free swimming cercaria some of these cells move so as to occupy the region which formerly contained the tanned cells (Fig. 4.3).

Before the formation of layer III has been completed, some of the keratin cells have started to concentrate below the muscle cells. When the secretion of the components of layer III is completed, the keratin cells push through the channels between the muscle cells so that while part of the cell, including the nucleus, still lies below the muscle cells, the remaining parts lie above the muscle cells and form a layer at the surface of the metacercaria (Fig. 4.36). The rods still contained within their vesicles appear to move towards the exterior surface where the vesicle membrane and the plasma membrane fuse, thus releasing the rod to the exterior (Fig. 4.37). This is a secretion process termed 'eccrine' by Kurosomi (1961), or more usually 'merocrine' (Ham and Leeson, 1961).

As the scrolls move towards the surface, they become less tightly wound. After passing through the plasma membrane, they remain within slight indentations in the cell surface, and each scroll then unwinds to form one of the lamellae of layer IV (Fig. 4.38). As successive rods are unrolled, each protein sheet is pressed firmly against the partly formed cyst wall to form the compact lamellar structure of this layer (see Chapter 3).

As far as can be ascertained, it appears that rods are released from all over the surface of the animal except for the regions of the oral and ventral suckers. This results in an even distribution of the rods which in turn leads to the formation of a keratinized layer of relatively uniform thickness. The only place where the keratinized layer shows any variation in structure is in the region of the ventral plug where the lamellae are less numerous and much less tightly packed (Chapter 3). During encystment, the ventral sucker remains approximately over what is to become the ventral plug and therefore in this region fewer rods would be secreted and hence, fewer lamellae would be laid down. It might then be expected that the ventral plug would have a much greater number of free "ends" than elsewhere, as seems to be so.

Although presumably individual cells have pushed their way to the surface, forming a layer overlying the muscle cells, no cell - cell contacts could be found in this layer. The cercaria seems to be bounded externally by one continuous plasma membrane which encloses a syncitial 'tegument', the nuclei of which lie below the muscle cells in invaginations of the inner plasma membrane. When secretion is completed, the syncitial tegument remains and it is still present in excysted juveniles.

In addition to the rod-like granules in keratin cells which appear to be moving towards the surface, there are often numbers of other secretory granules which in some cases appear identical to the mucopolysaccharide and mucoprotein granules (Figs. 4.33; 4.34). The manner in which these granules enter the rod-producing cells remains obscure. Both SH groups and -S-S- bonds were demonstrated in the rods. It is possible that the disulphide bonds occur between each member of the double sheet and the sulphydryl groups lie on the outer edges of the double sheet. This grouping would allow the scroll to unroll and when the lamellae are formed, the free thiol groups could be oxidized to form more disulphide bonds. Between the lamellae of the partly formed keratinized cyst wall, small vesicles can be distinguished (Fig. 4.38). Their function is unknown but they could

represent the agent effecting oxidation of free sulphydryl groups. Alternatively, perhaps this is due to atmospheric oxidation.

It seemed likely that the muscular rotatory movements of the metacercaria during encystment play some part in unrolling of the rods. During the secretion of the keratin material, the metacercaria is in the third phase of movement when the whole surface of the body is in continuous flowing motion (Fig. 4.24 l, m). To test this hypothesis, some metacercariae were anaesthetized with a 0.1% solution of chlorbutol in tap water 30 min. after encystment had begun, and fixed 1 hr. later. Examination of sections of these cysts showed that although secretion of some rods had occurred, they had not unrolled (Fig. 4.39). How unrolling takes place is not clear. Probably the free end of the scroll becomes attached to the adjacent lamella of the cyst wall and then, while the scroll is held in a depression in the surface of the metacercaria, the rotatory movement of the animal draws the body of the scroll away from the free end, thereby causing it to unroll.

The origin of the protein-lipid matrix in which the lamellae are held (Chapter 2) is not known. It has been noted, however, that in the keratin cells, another

Fig. 4.25: Electron micrograph. An early cercarial embryo within the redia containing a very few nuclei (n). The embryonic epithelium (ee) completely encloses the embryo and is joined by a strand of tissue (as) to the redia body wall (rw). The embryonic epithelium rests on a basement membrane (bm). In the lower left hand corner is another more mature cercaria also contained by an embryonic epithelium (arrow). r, reticulum. Osmium tetroxide, Millonig's lead.

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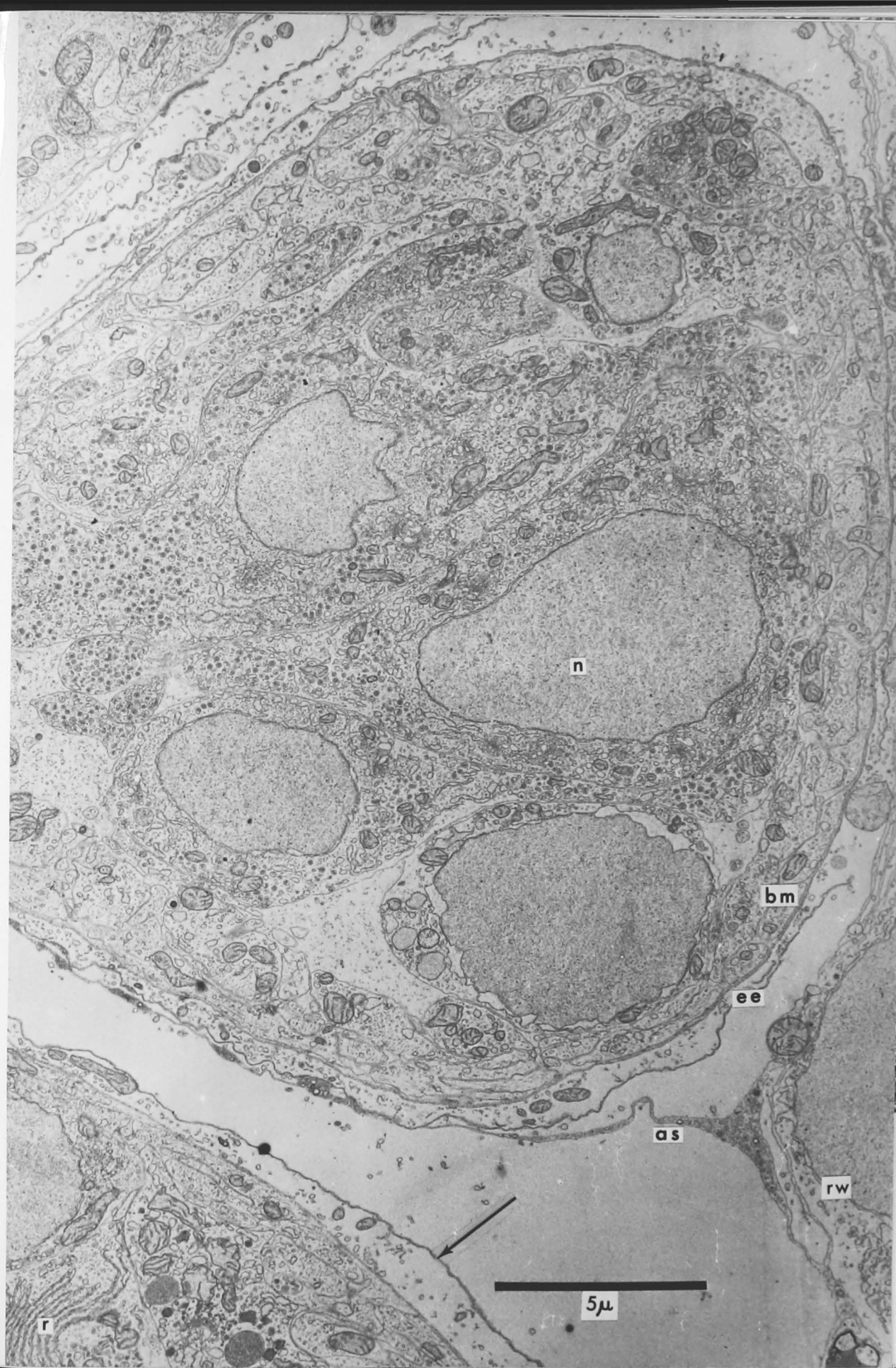


Fig. 4.26: Electron micrograph. The edge of a cercaria within a redia showing the attachment of a protoplasmic sheet (arrow) to the embryonic epithelium (ee). The other end of the sheet is attached to the redia body wall. m, mitochondrion; ks, keratin scroll. Potassium permanganate, Millonig's lead.

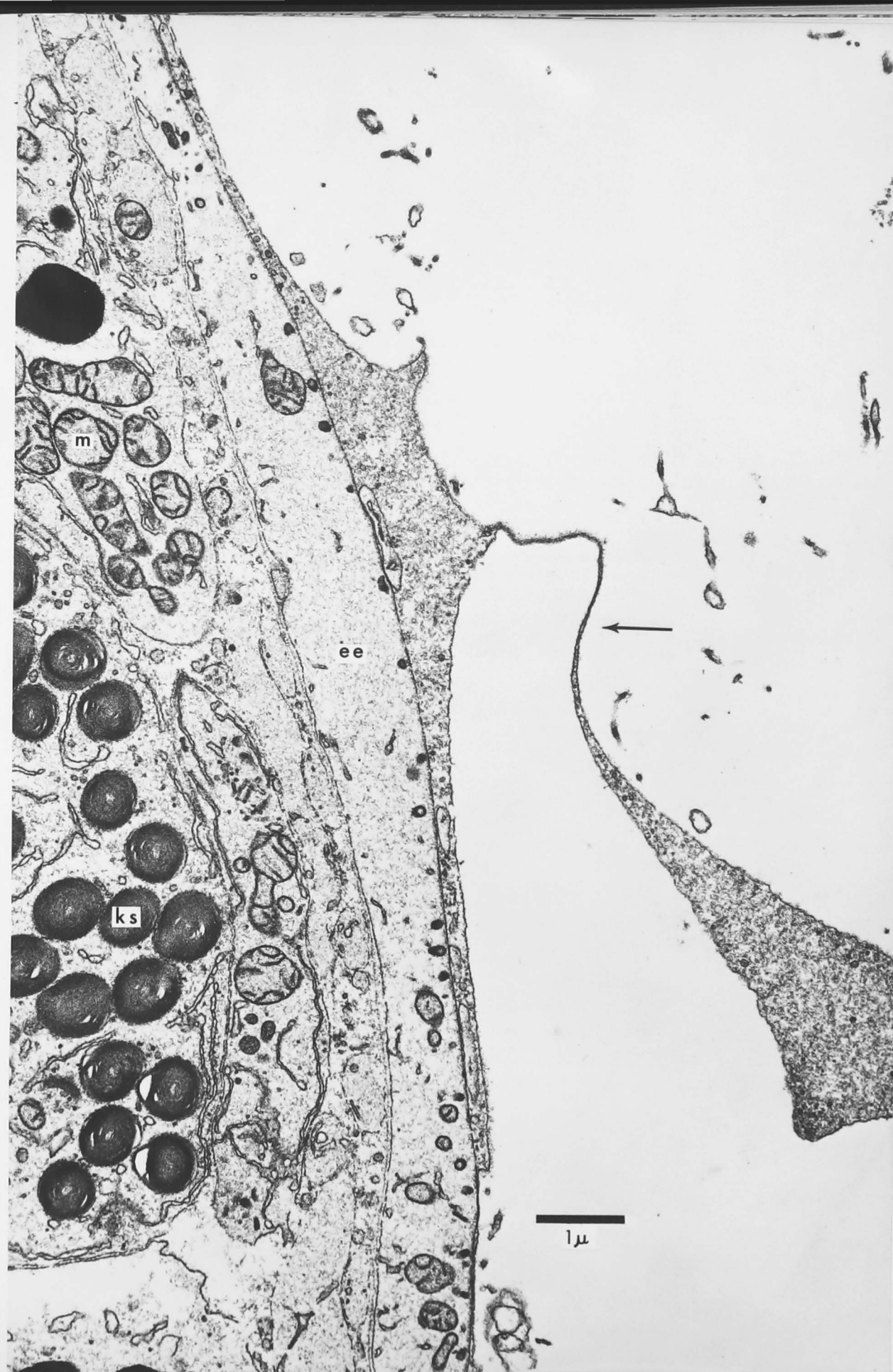
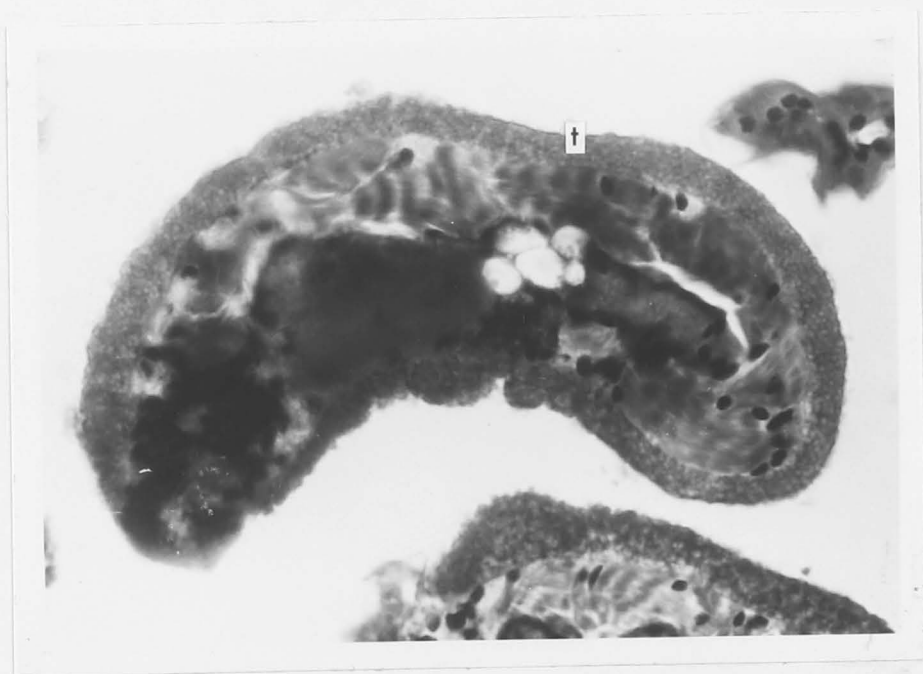


Fig. 4.27: Light micrograph. L.S. cercaria within snail.

The tanned protein material (t) selectively stained with aqueous BPB is now outside the cercaria. Compare Fig. 4.4. BPB, neutral red.



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Fig. 4.28: Electron micrograph. The embryonic epithelium (ee) is filled with granules (g), some of which appear similar to those of the mucopolysaccharide cells (Figs. 4.12; 4.13). Others are not enclosed within membranes and have a denser exterior and may be mucoprotein granules (arrow). The prominent invagination of the outer plasma membrane (pm) may function in pinocytosis or may be a remnant of a transverse portion of the plasma membrane of one of the cells of the embryonic epithelium.

mc, muscles; m, mitochondrion; rw, redia body wall. Osmium tetroxide, Millonig's lead.

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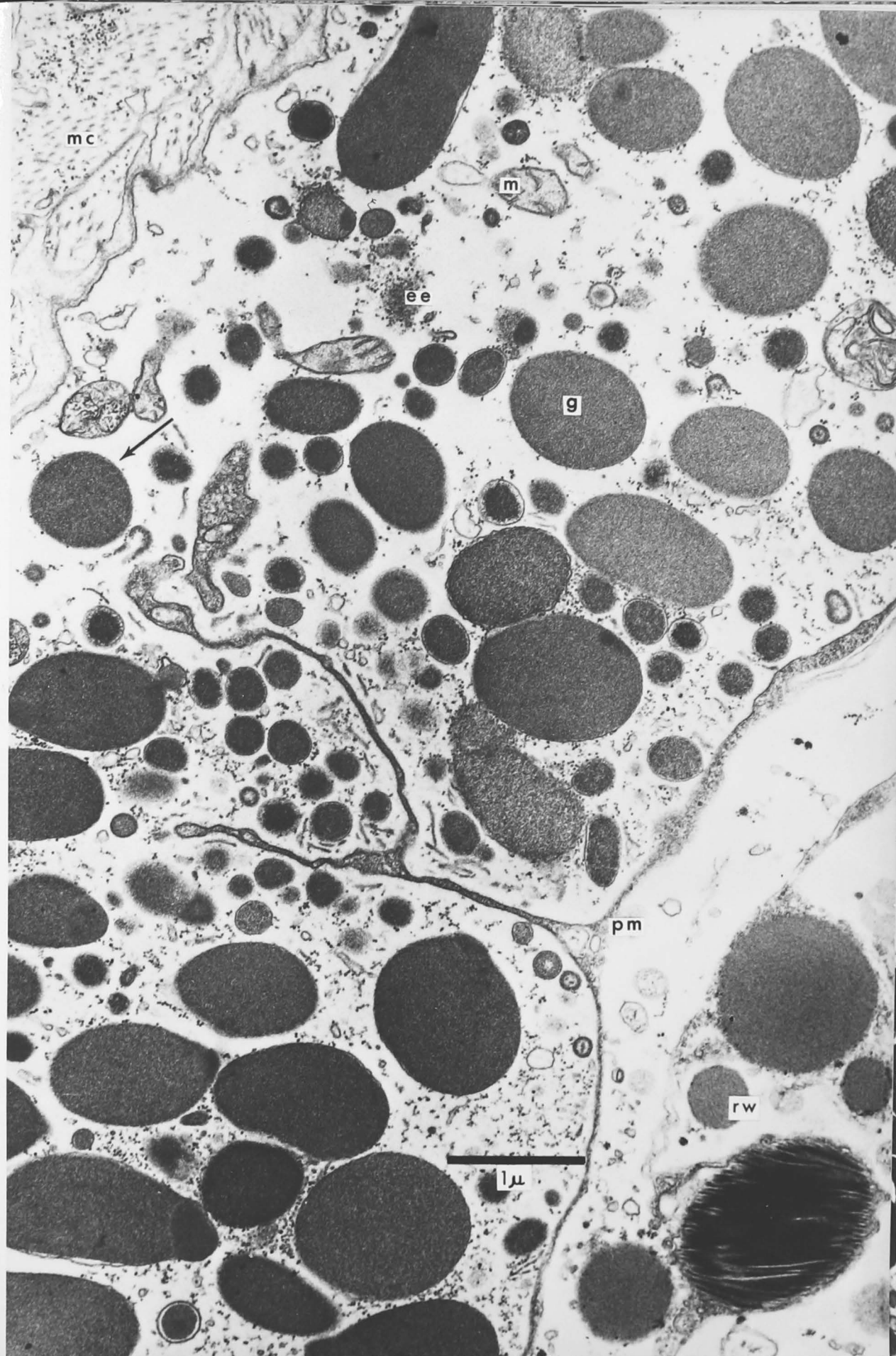


Fig. 4.29: Electron micrograph. The edge of a free swimming cercaria. The embryonic epithelium (ee) is distended with tanned protein granules (tg) and carbohydrate-protein granules (g) which are concentrated more towards the inner surface. The keratin cells containing scrolls (ks) are very near the surface. pc, mucopolysaccharide cell; mc, muscles; pm, plasma membrane. Potassium permanganate, Millonig's lead.

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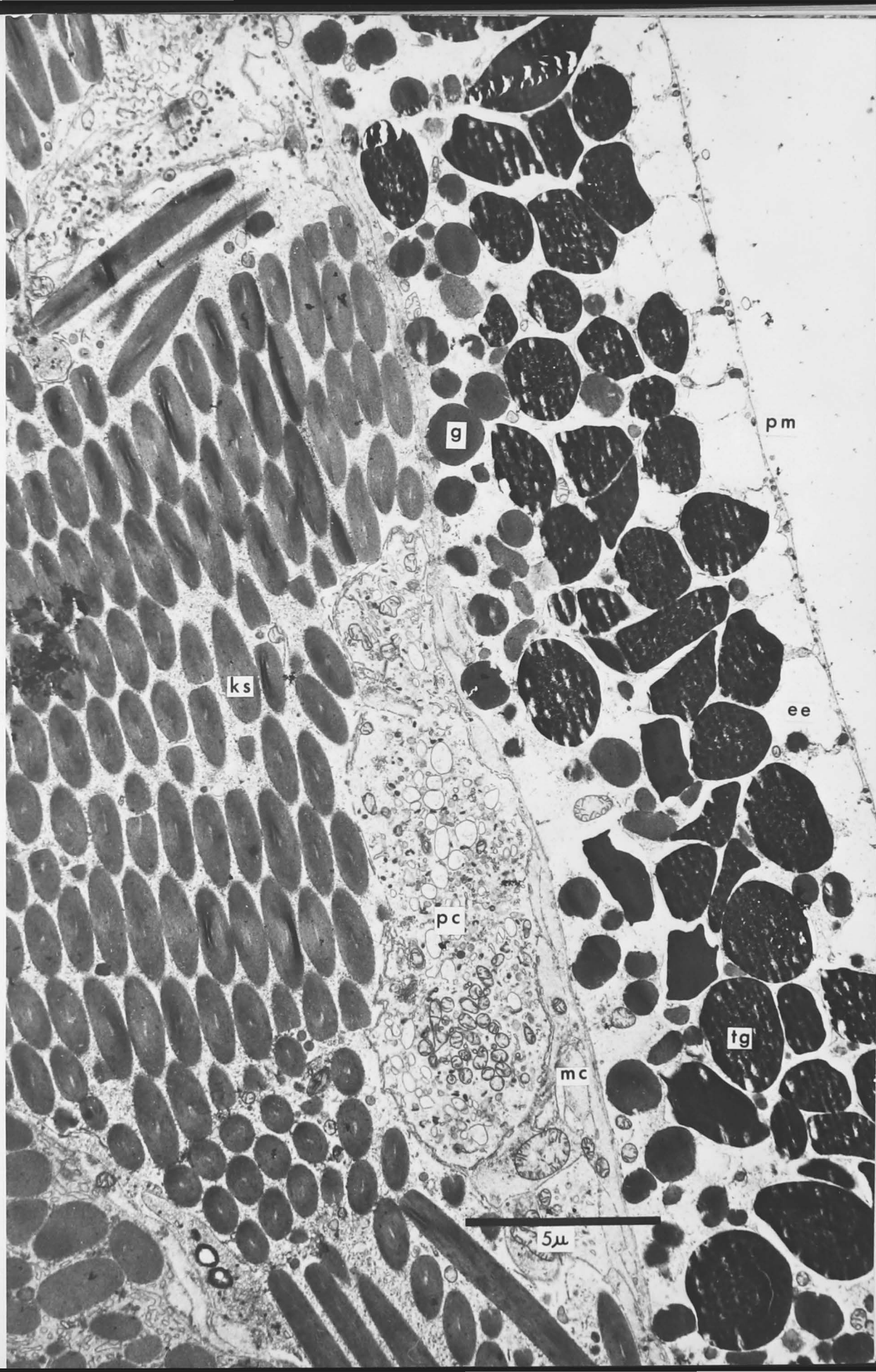


Fig. 4.30: Electron micrograph. The contents of the tanned protein cells including the secretory granules (tg), mitochondria (m) and reticulum (r) are flowing into the embryonic epithelium (ee) through a channel between the muscle cells (mc) which is lined by basement membrane (bm). Some elements of the reticulum are contained within smooth surfaced vesicles and may be in the process of being resorbed. n, nucleus. Osmium tetroxide, Millonig's lead.

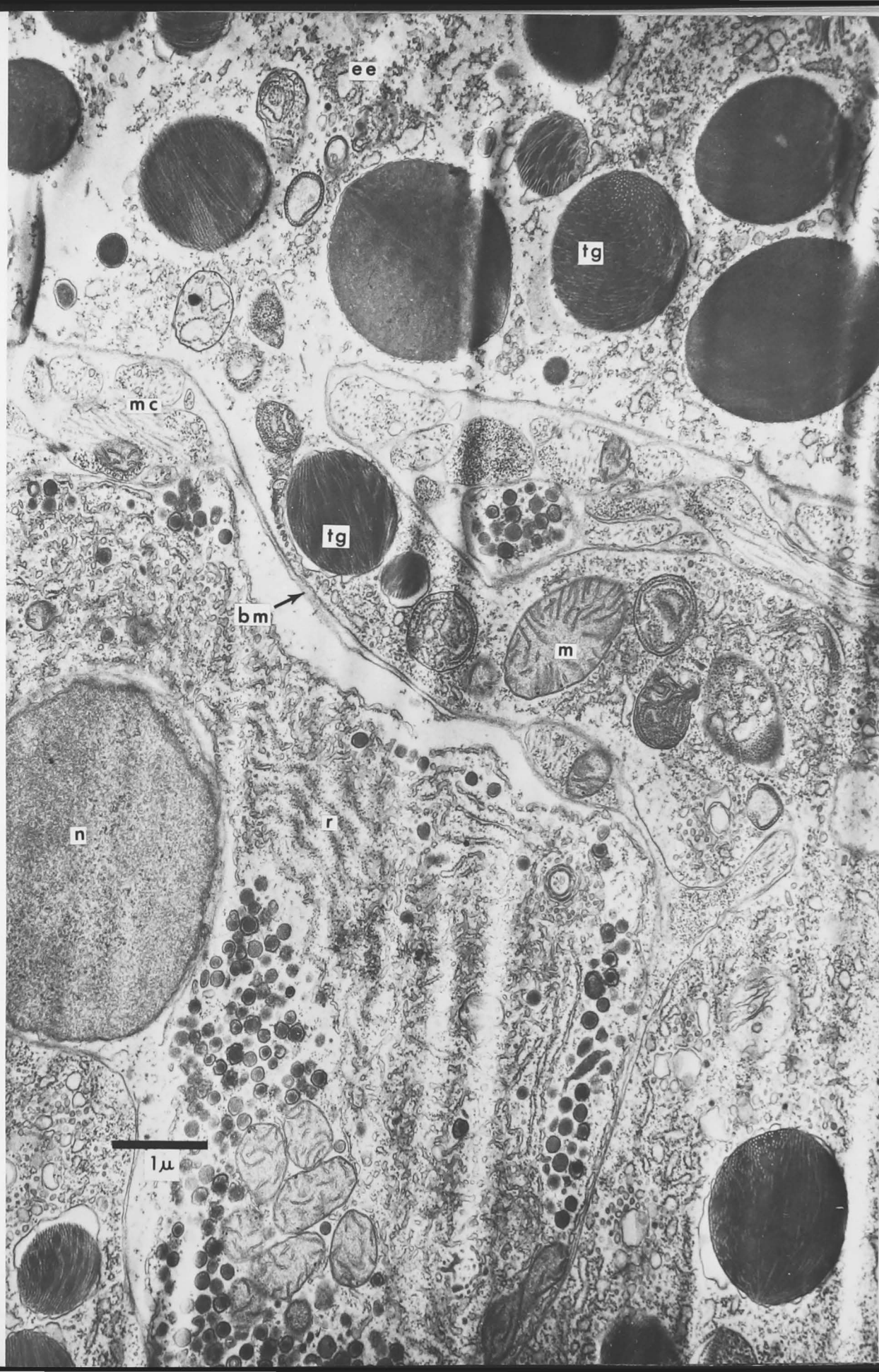


Fig. 4.31: Electron micrograph. The embryonic epithelium (ee) is distended with tanned protein granules (tg) and carbohydrate-protein granules (g). The granules of a mucoprotein cell (mp) appear to be about to move through a channel (arrow) between the muscle cells (mc) which is lined by the basement membrane. pc, peripheral mucopolysaccharide cells; n, nucleus. Potassium permanganate, Millonig's lead.

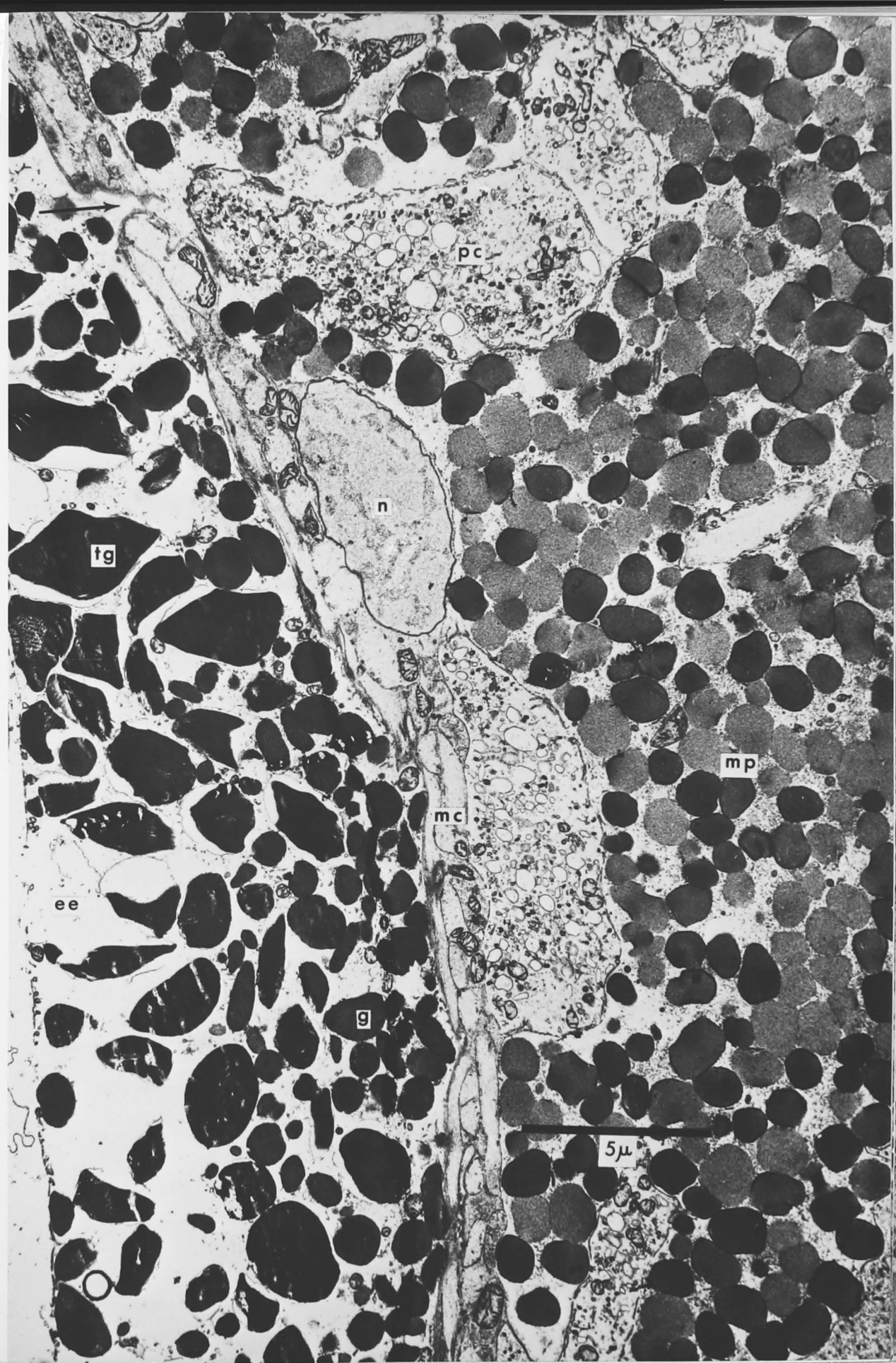


Fig. 4.32: Electron micrograph. Section of a cyst 10 min.

after encystment has begun. A jumbled collection of granules (g) lies between the body of the cercaria and the fibrous elements of Layer II (II). The granules are of two types, the denser ones resembling those seen in the peripheral mucopolysaccharide cells (Fig. 4.9). The less dense granules have a characteristic fine structure. Some mitochondria (m) occur between the granules. The inner plasma membrane is visible in some places (arrow) but the outer plasma membrane is not. mc, muscles. Osmium tetroxide, Reynolds' lead.

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Fig. 4.33: Electron micrograph. Layer IIIa has been formed although some consolidation is still to take place. Layers IIIb and c are in the process of formation and some mitochondria and elements of the reticulum are still present among the finely granular matrix. A large secretory granule similar to those within the keratin cell (g) is also present and a plasma membrane (pm) forms the outer boundary. mc, muscle cell; ks, keratin scroll. Osmium tetroxide, Reynolds' lead.

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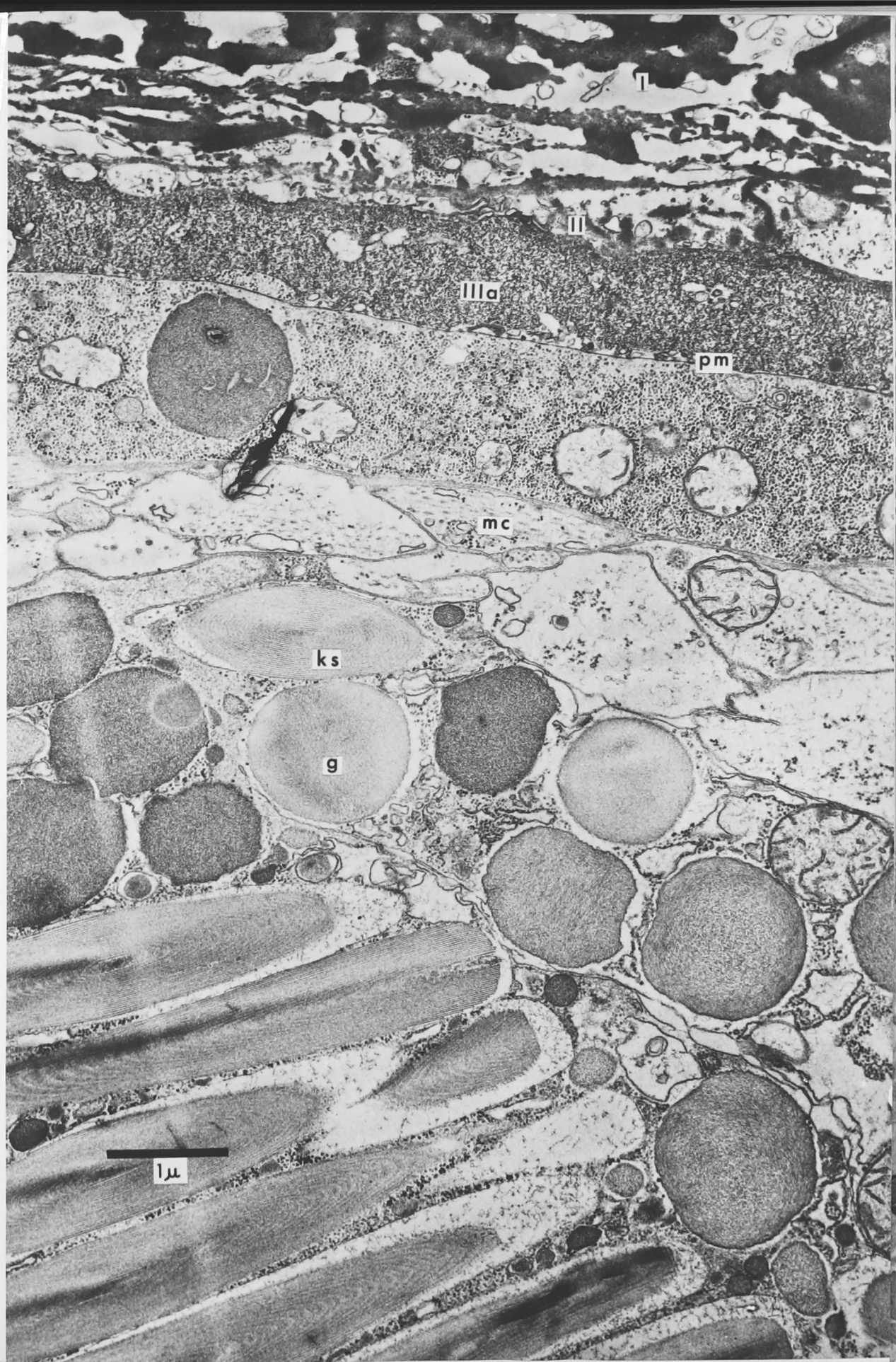
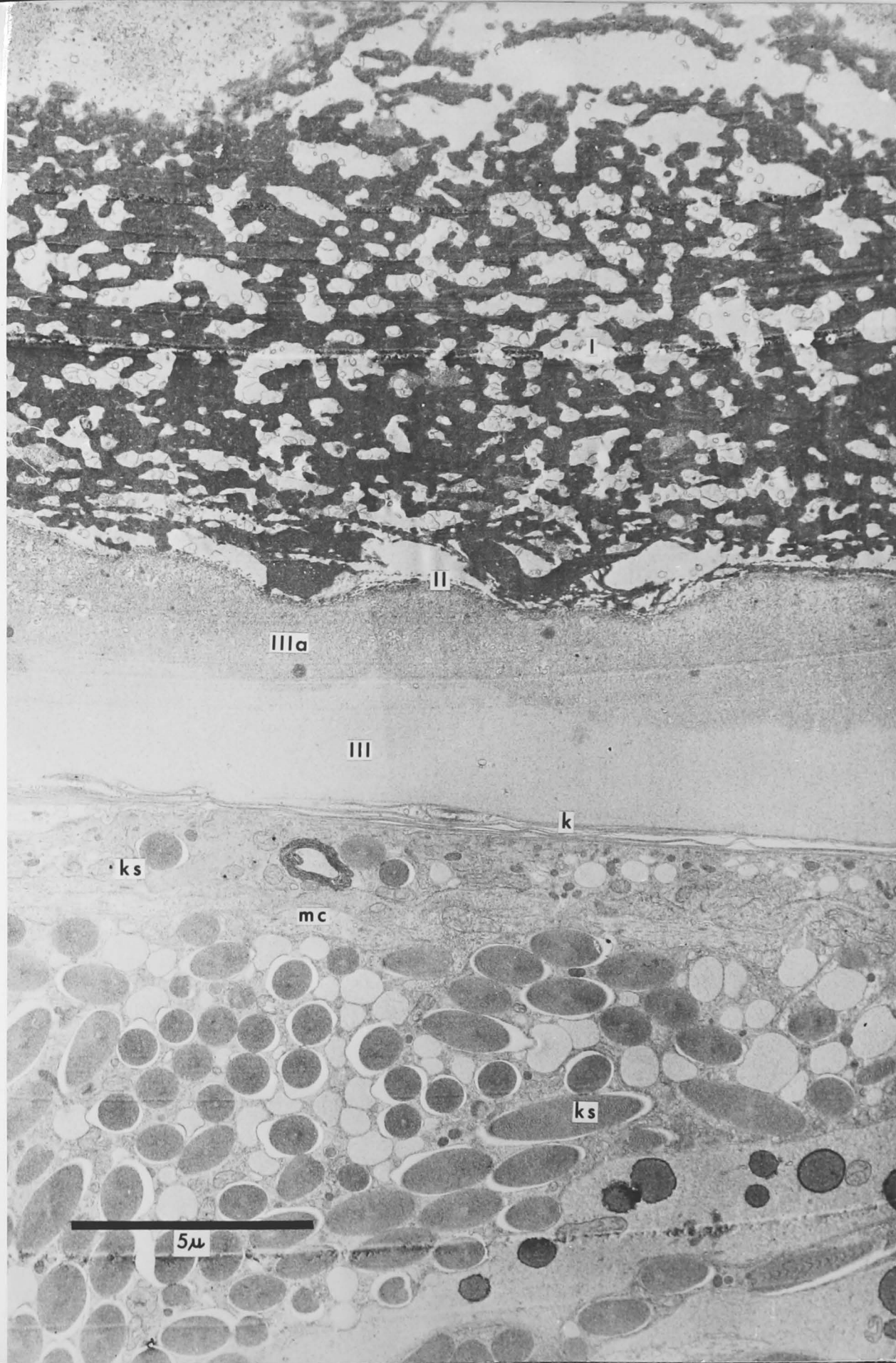


Fig. 4.34: Electron micrograph. The secretion of Layer III has finished and the first lamellae (k) of Layer IV have been formed. Layer III still contains a few small degenerate vesicles. Part of the keratin cell has formed a layer outside the muscle cells (mc) and keratin scrolls (ks) are visible either side of the muscles. Layers I and II are fully formed. Osmium tetroxide, Millonig's lead.



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Fig. 4.35: Electron micrograph. Partly formed cyst. The process of secretion of the mucopolysaccharide granules is holocrine and only the remnants of the cell remain. n, nucleus; m, mitochondrion; pm, plasma membrane; rb, ribosomes; IIIa, Layer IIIa. Osmium tetroxide, Millonig's lead.

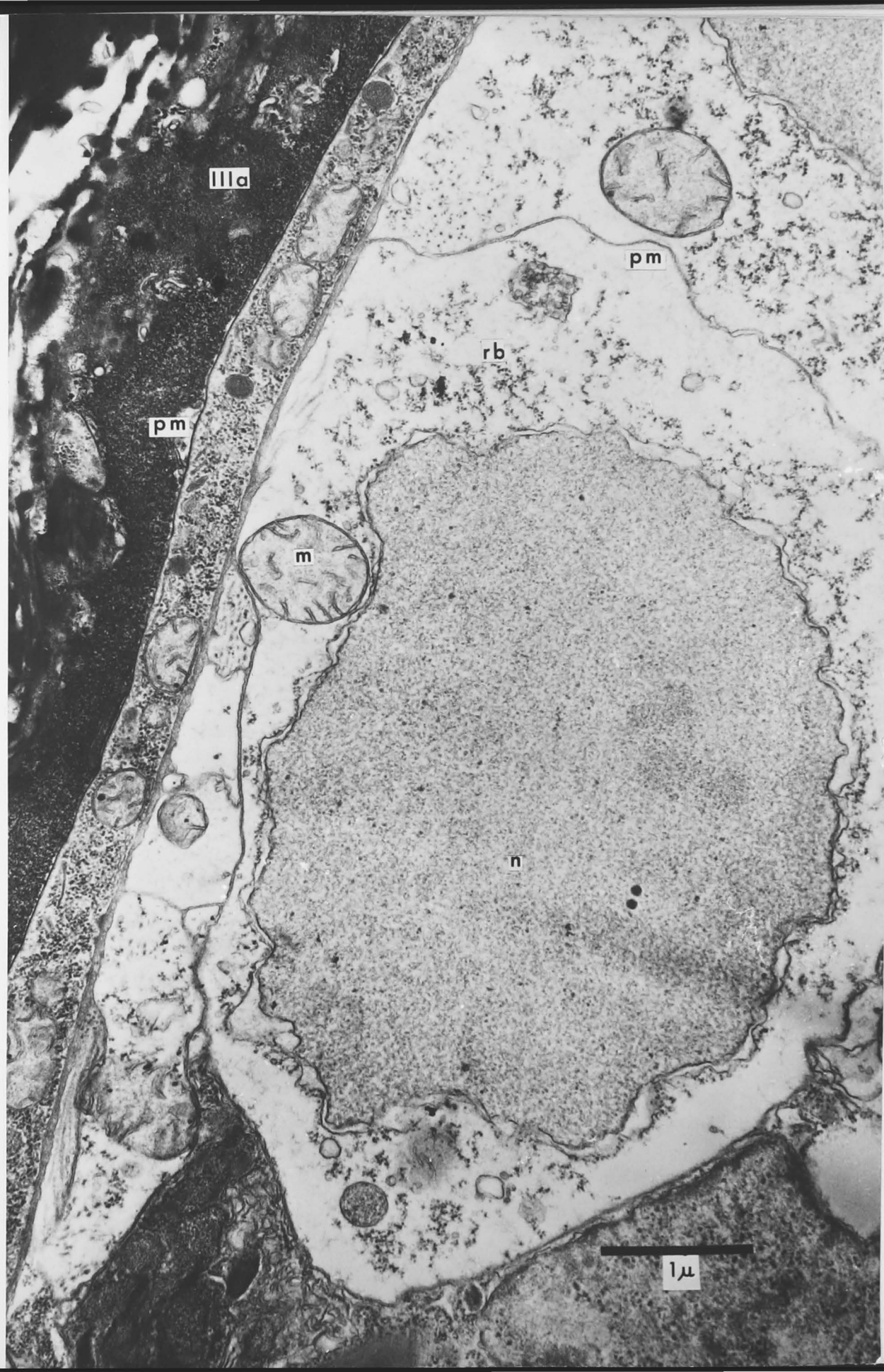


Fig. 4.36: Electron micrograph. The formation of Layer III is virtually completed and the first lamellae of the keratinized Layer IV (k) have been laid down. A cytoplasmic layer containing mitochondria and other organelles lies outside the muscle cells (mc) but is still connected to the interior through a channel (arrow) between the muscle cells. A keratin scroll (ks) lies roughly parallel to the surface. Osmium tetroxide, Millonig's lead.

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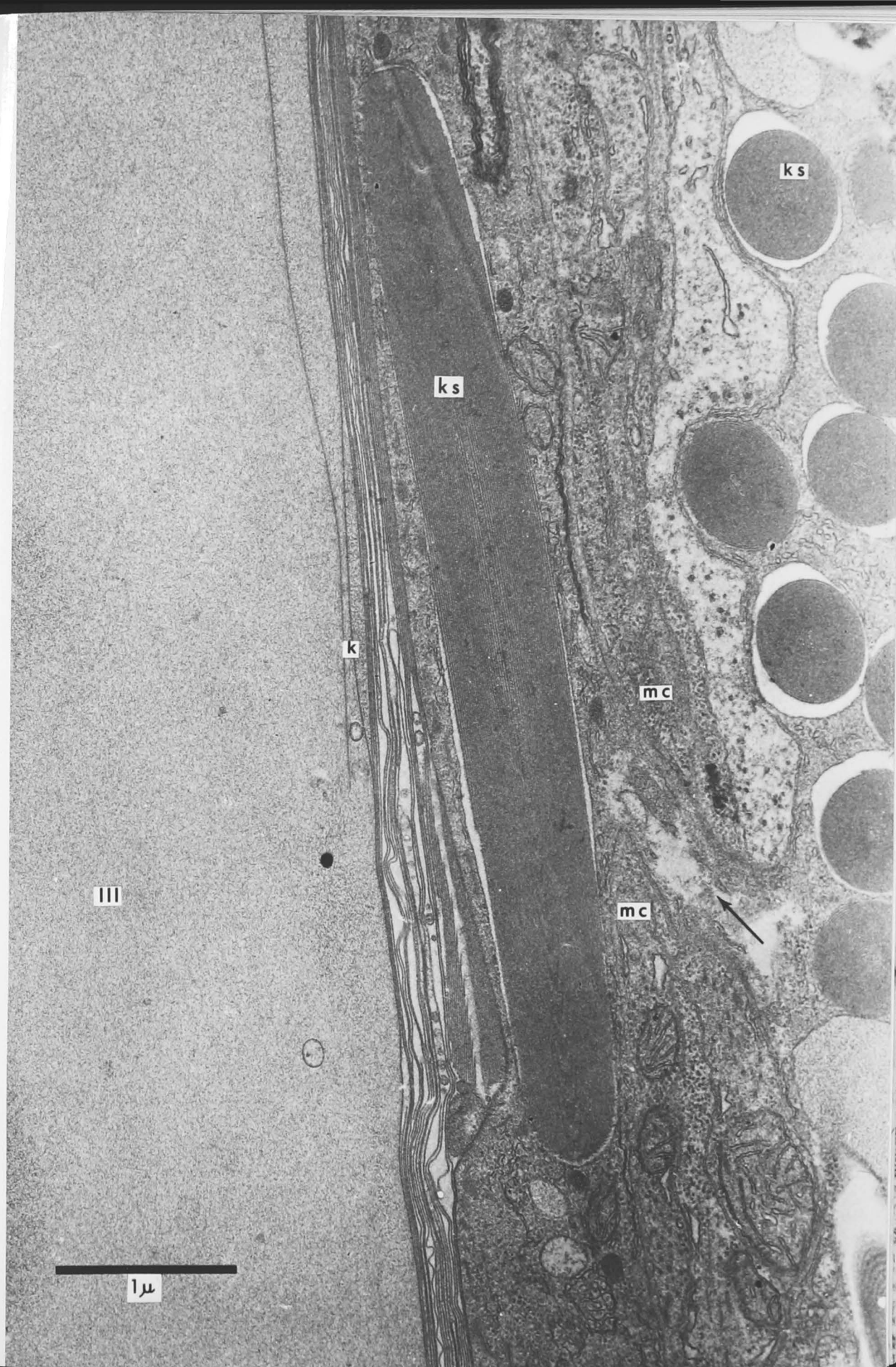


Fig. 4.37: Electron micrograph. Partly formed cyst. The formation of Layer IV has just begun and some of the keratin lamellae (k) are visible. Part of the cytoplasm of the keratin cell forms a layer external to the muscle cells (mc) and the nuclei (n) remain below. As the keratin scrolls (ks) approach the surface they become less tightly rolled. ka, kb are successive stages in this process. The vesicle membrane surrounding the scrolls fuses with the plasma membrane (pm) releasing the scroll (kc) to the exterior, but it continues to be retained within a depression in the surface of the animal. Osmium tetroxide, uranylacetate.

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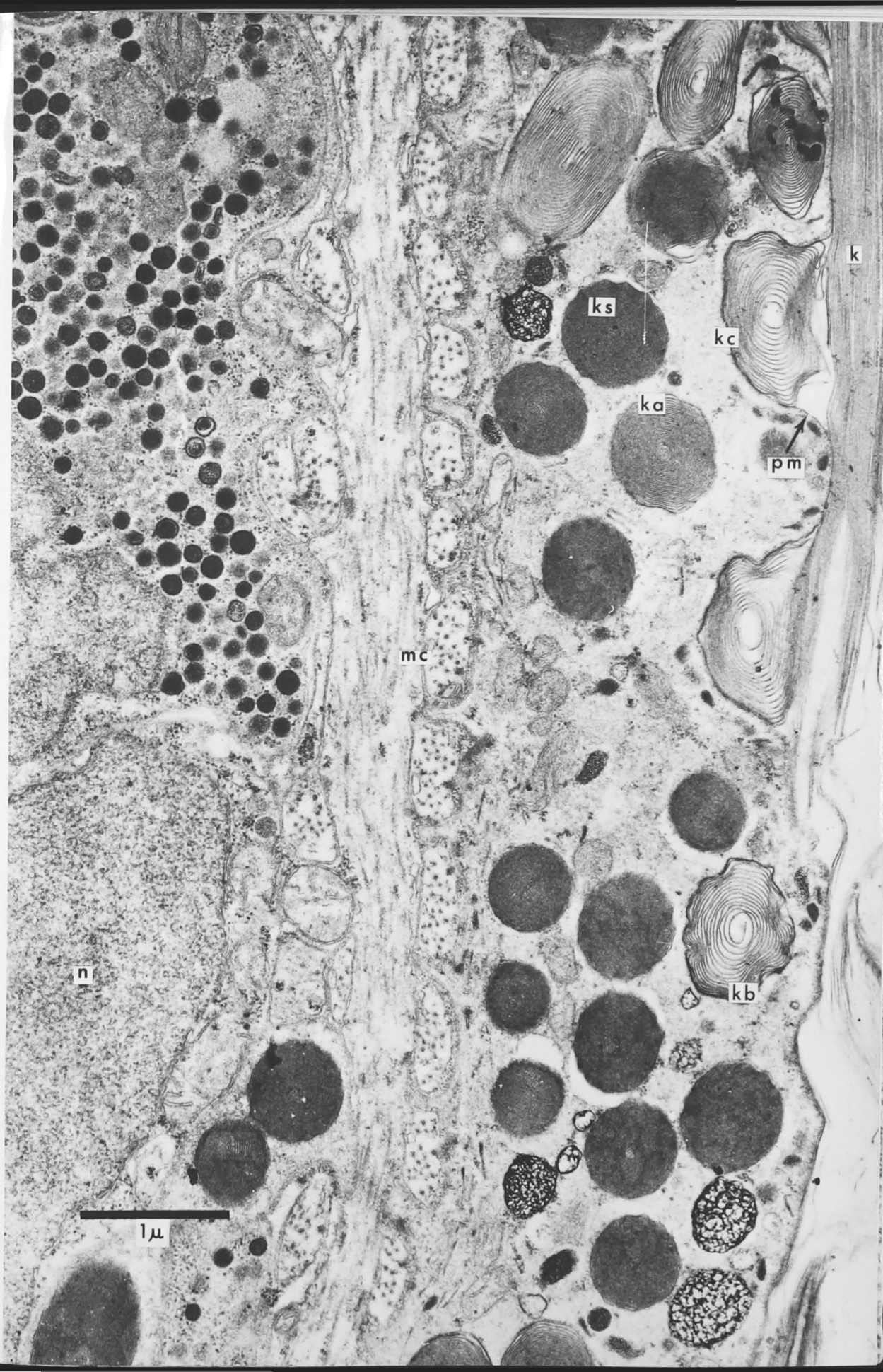


Fig. 4.38: Electron micrograph. The unrolling of a keratin scroll (ks) into a sheet which will form a lamella of the keratinized Layer IV (k) which consists of numerous closely packed lamellae. There is a cytoplasmic layer outside the muscle cells (mc) which is bounded externally by a plasma membrane (arrow). m, mitochondrion. Osmium tetroxide, Millonig's lead.

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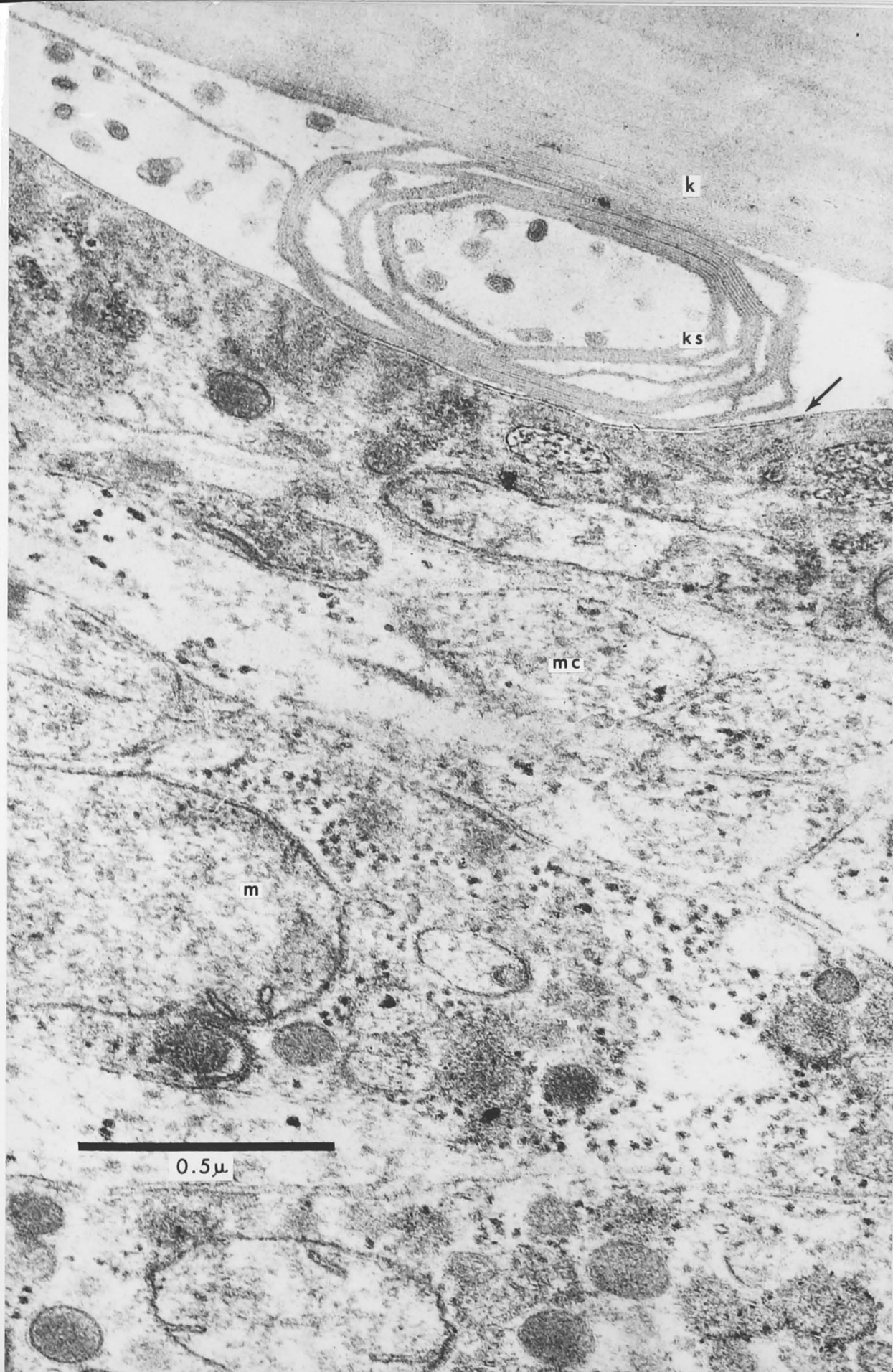


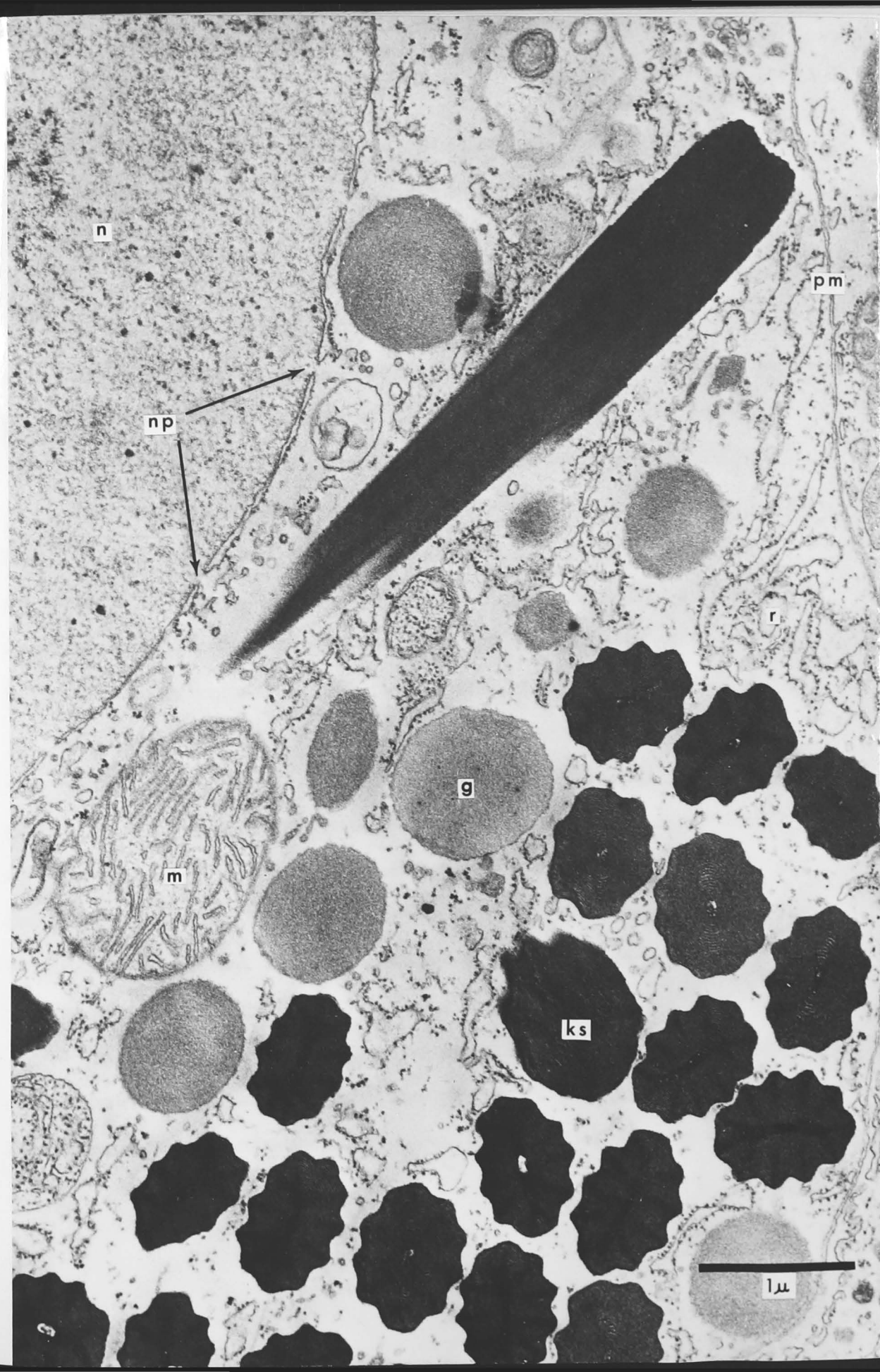
Fig. 4.39: Electron micrograph. Section through the outer edge of a metacercaria which was anaesthetized during the secretion of the keratin scrolls (ks). Numerous scrolls (arrows) are visible between the edge of the animal and Layer III of the cyst wall (III). m, mitochondrion. Osmium tetroxide, Millonig's lead.

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Fig. 4.40: Electron micrograph. The keratin cell contains another type of secretory granule (g) in addition to the keratin scrolls (ks). n, nucleus; np, nuclear pore; pm, plasma membrane; r, reticulum; m, mitochondrion. Osmium tetroxide, Millonig's lead.

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structurally distinct granule is also present (Fig. 4.40) and perhaps these contribute to the matrix.

DISCUSSION

It has been shown in these studies, that the cercaria contains four types of secretory cells, the contents of which are used in forming the cyst wall. The cells have been characterised histochemically and electron microscopically.

The tanned protein cells occupy almost all of the ventral half of the body and contain granules which give positive reactions to tests for basic protein and phenols, and which appear in electron micrographs as dense ovoid structures contained within vacuoles and traversed by less dense channels.

The mucopolysaccharide cells are located peripherally and contain acid and neutral mucopolysaccharides. In the electron microscope, the secretory granules are contained within vesicles and appear as dense elongate bodies with a small, less dense, sphere near one pole. Granules of different appearance are also present but probably represent immature stages of the first kind of granule.

The mucoprotein cells lie more or less centrally between the ventral sucker and the pharynx. They contain a granular mucoprotein substance which appears in the electron

microscope as a number of closely packed granules of medium density, not contained within a vesicle and which often appear broken.

The keratin cells fill almost the whole of the dorsal half of the body and contain bundles of parallel rod-shaped granules about $5\ \mu \times 0.5\ \mu$. The rods are composed of a protein which contains both free sulphhydryl groups and disulphide bonds. The rods have the appearance of a protein sheet which has been tightly rolled to form a scroll; at high magnifications, the sheet proved to be composed of a double layer.

Although previous to the present study the secretory cells of a large number of cercariae have been studied extensively using light microscopy, classical histological techniques and histochemistry have been used only sparingly, and investigations using the electron microscope have not been reported at all. The tanned protein cells are undoubtedly those which have been described in this cercaria as "cystogenous" cells by other authors (Thomas, 1883; Wright, 1927; Bovien, 1934; Rees, 1932). They are similar in structure and position to "cystogenous glands" reported in some other cercariae (Barlow, 1925; Swales, 1935; Rothschild, 1936; Lengy, 1960). The studies reported here have shown

that this terminology is imprecise as other cells also contribute their products to the cyst wall.

The chemical nature of the secretory product of the tanned protein cells has not been determined previously, although Campbell (1960) demonstrated the presence of phenolase in the cercariae of Fascioloides magna and inferred from this that the secretion was a tanned protein. It is possible that glands of similar appearance and position in other cercariae also contain tanned protein since the outer cyst walls appear to have similar properties to the Fasciola outer cyst wall (Chapter 2).

The fine structure of the granules resembles to some extent the fine structure of the vitelline cells of the adult Fasciola (Bjorkman and Thorsell, 1963). These are also known to be concerned in a tanning process (Smyth and Clegg, 1958).

Some tanning systems have been described in the Turbellaria (Smyth and Clegg, 1958) but in those cases they are also concerned with the formation of the egg-shell.

The peripheral mucopolysaccharide cells have not previously been described in the Fasciola cercaria. They correspond in position to the mucous gland cells which lie near the dorsal and ventral surfaces of Cercaria purpurae (= Parorchis acanthus) (Rees, 1937). They also bear some

resemblance in location and their histochemical reactions to the "mucoid" glands found in the cercariae of many different families (Kruidenier, 1951, 1953a, b, c; Kruidenier and Mehra, 1957; Ito and Watanabe, 1958a, b; Ortigoza and Hall, 1963). All these authors have observed cells which stain metachromatically with toluidin blue and which occur mainly on the ventral surface although in some cases they are found dorsally and laterally.

In some turbellaria, glands containing acid mucopolysaccharide material occur on the ventral surface (Skaer, 1961; Pedersen, 1963). Glands of this type are common in other animals where it is thought that their secretions act as lubricants.

The fine structure of the granules in turbellaria (Török and Röhlich, 1959; Skaer, 1961; Pedersen, 1963) resembles that of the mucopolysaccharide granules described here, although this has little significance because they lack a distinctive structure. In the mature state the turbellarian granules have a banded appearance similar to that of one kind of granule in the Fasciola cercaria (Fig. 4.32).

The mucoprotein cells occupy a position corresponding to the "salivary gland cells" of Rothschild (1936) and to the "penetration glands" of Rees (1937). Both these authors reported ducts opening anteriorly although Rothschild had

great difficulty distinguishing them. In the F. hepatica cercaria, these glands have not been previously described, and ducts could not be found either with the light microscope or with the electron microscope.

The cells are also similar in position and show some chemical similarity to the penetration glands of schistosomes and other cercariae. The increasing volume of literature on glandular secretions of this nature has been recently reviewed (Stirewalt, 1963).

The fine structure of the granules is again not distinctive, but they look somewhat similar to the goblet cell granules in the rat jejunum (see Palay, 1958).

Cells which contain rod-like granules have been reported from the cercariae of other families besides the Fasciolidae, e.g. Paramphistomidae (Sonsino, 1884; Krull and Price, 1932; Durie, 1953; Lengy, 1960), Notocotylidae (Martin, 1956; Singh and Lewert, 1959), Echinostomidae (Cardell, 1962), Philophthalmidae (Rees, 1937), but their chemical nature has not previously been ascertained. In the cercaria of Himasthla quissetensis, Cardell (1962) has described rod-like granules ("bacilliform rods") which have a similar fine structure and Dixon and Mercer (1965b) have observed almost identical structures in the cercaria of Parorchis acanthus. The rods in section bear a superficial resemblance to

phospholipid spherulites (Mercer, 1962), but the spherulites are thought to be concentric shells, not spirals, and of course, there is no chemical similarity.

There are some resemblances between the rods of cercariae and the rhabdites of Turbellaria. Although there has been a considerable amount of recent work on rhabdites, the chemical properties and fine structure are not quite certain. Thus Skaer (1961) and Pedersen (1963) disagree on whether sulphhydryl groups are present or not. However, since Pedersen has obtained a positive result using a more precise histochemical test (DDD reagent) it seems likely that thiol groups are present. Pedersen (1963) has described the fine structure of the rhabdites as either homogeneous or granular. Skaer (1961) finds that in Polycelis nigra, early stages in the formation show an internal fine structure consisting of longitudinal fibres 120 A wide and spaced 300 A apart. At a later stage, granules are deposited on the fibrils and deposition continues until the original fibrous structure is obscured. In longitudinal section, the cercarial rods appear fibrous with some very small granular bodies on the 'fibres'. Clearly, it would be interesting to study the very early stages of rhabdite formation in some detail in order to determine if the structures are in any way similar to those in the cercariae.

The Formation of the Cyst Wall

(a) The outer cyst wall

The secretion of the outer cyst wall components takes place just before the cercaria leaves the redia. The contents of the tanned protein cells, some of the granules of the peripheral mucopolysaccharide cells and probably some of the granules of the deeper mucoprotein cells are transferred into the embryonic epithelium, a thin layer one cell thick, on the outside of the cercaria. Stratification of the granules occurs so that the tanned protein material is located mostly in the outer regions of the embryonic epithelium and the granules of carbohydrate-protein material occupy the inner regions next to the cercaria. At encystment, the cercaria appears to press the ventral part of the embryonic epithelium against the substrate to which it adheres and the cercaria then contracts vigorously, shedding the embryonic epithelium with the contained granules. The tanned protein granules then tend to dissociate and adjacent granules adhere to form a meshwork. The carbohydrate-protein granules are drawn into long, poorly defined fibres, presumably by the initial rotatory movements of the metacercaria.

Langeron (1920) described in some detail the formation of a thin membrane formed of large flat cells with voluminous projecting nuclei, which enveloped Cercaria vivax embryos in

the redia. He believed that the "primitive epithelium" is cast off when "the subcuticular cells...commence to secrete the cuticle of the young cercaria".

In the cercaria of F. hepatica, a similar cellular layer is formed at a very early stage in development but is not cast off until the cercaria commences to encyst. It is during the process of encystment that the metacercarial "tegument" is secreted (see later) so there is this further point of similarity with C. vivax. In this investigation, the term 'embryonic epithelium' has been proposed in preference to "primitive epithelium" (Dubois, 1929; Rothschild, 1936) to denote the early formation of this layer and its position at the surface of the cercaria.

In the Fasciola cercaria the embryonic epithelium is difficult to detect using light microscopy. It was only faintly visible in paraffin sections. Rees (1937) stated categorically that a "primitive epithelium" does not exist in the cercaria of Parorchis acanthus. However, using the electron microscope, Dixon and Mercer (1965b) have observed that the formation of the outer cyst wall in this cercaria closely parallels that in the Fasciola cercaria and that an embryonic epithelium is present. In the only other observations made on the surface of cercariae using electron microscopy, Cardell (1962) has found that a cellular 'cuticle'

overlies the muscle cells of the cercaria of Himasthla quissetensis. Cardell's micrographs of the surface layers show a striking similarity to the 'tegument' of excysted juvenile F. hepatica observed in these studies and therefore suggest that observation of earlier stages in cercarial development could demonstrate the presence of an embryonic epithelium.

In the earlier descriptions of the cercariae of F. hepatica a minor point of difference arose concerning the location of the "cystogenous cells" (= tanned protein cells) in the mature cercaria. Thomas had originally described them as located in two distinct lateral areas but other authors (Wright, 1927; Rees, 1932; Bovien, 1934; Wesenberg-Lund, 1934; Kruidenier, 1953a) found that the "immature" cercariae had such an appearance but in the "mature" cercariae, the contents of these cells formed an enveloping layer over the surface. Kruidenier (1953a) was the only one to connect this specifically with cyst formation.

According to Bovien (1934) the secretion of the granules is completed in the redia and the results reported here would confirm that this is generally so. Bovien also believed that the granules gather in a space between two layers of the "cuticula" (sic). A similar process was described by Rothschild (1936) in an unnamed cercaria, although she

believed that the granules were released initially into the outer layer of the "cuticula", which burst due to the pressure of the granules thus releasing them into the "primitive epithelium".

The secretion of a "mucoid" film around cercariae before their emergence from the snail, has been shown to be a common phenomenon (see Stirewalt, 1963). In most cases, the secretion originates from glands localised on one particular surface, usually the ventral, but the film covers the entire surface of the body. Kruidenier (1953b) has attempted to explain the adherence by postulating a change in the viscosity of the granules after secretion, but he does not consider how ventral glands are able to form a layer on the dorsal surface. According to Kruidenier and Mehra (1957) a distinct "cuticula" is present in xiphidiocercariae and in pronocephalid cercariae prior to discharge of the metachromatic glands. It is not clear what structure was designated as "cuticula" by Kruidenier and Mehra (1957) but it is possible that these cercariae possess an outer cellular layer corresponding to the embryonic epithelium of the Fasciola cercaria. In an analogous way, the contents of the metachromatic glands may be secreted into this layer, which thus limits the spread of the granules outwards, forcing them up over the dorsal surface.

In the Fasciola cercaria, the tanned protein granules and the mucoprotein granules are secreted into the embryonic epithelium through channels between adjacent muscle cells. The basement membrane is not continuous around the body, but encloses groups of muscle cells, and presumably the secretory channels occur between these groups and thus appear to be lined by the basement membrane.

The physiological processes associated with the shedding of the embryonic epithelium are not known. It does seem likely however, that the strong muscular contraction of the metacercaria plays an important part. The initial flattening of the cercaria is thought to cause adherence of the ventral region of the embryonic epithelium to the substrate. No evidence of any adhesive secretion from ventral 'mucous' glands could be found. It seems reasonable to assume that, as well as causing adherence to the substrate, the flattening of the cercaria would squeeze out the contents of the ventral regions of the embryonic epithelium towards the periphery, thus forming the characteristic flared basal regions of the outer cyst wall (Fig. 2.4).

It has been noted that the tanned protein granules tend to break up and adhere to form the loose meshwork which is characteristic of the finished layer I. Nothing is known of the factors responsible for this happening, although it

should be noted that the adherence of granules is not necessarily due to the tanning process; a less stable form of linkage could be involved.

(b) The inner cyst wall

After the outer cyst wall has been laid down, it acts as a mould in which the inner cyst can be formed. Mucopolysaccharide granules from the surface cells and mucoprotein granules from the deeper cells are secreted to the surface and then some stratification occurs resulting in the formation of the sub-layers IIIa, b and c. It has been noted that the distribution and relative widths of the sub-layers is variable (Chapter 2). This can be related to the apparent lack of any firm control over the secretion and formation of these layers.

At about the time when the protein-carbohydrate secretions have been exhausted, the rod-containing cells extend part of their cytoplasm through channels between adjacent muscle cells and form a compact layer containing rods and cellular organelles outside the muscle cells. The rod-like granules are secreted through the limiting plasma membrane to the exterior, where each rod is unrolled to form a sheet of protein which is pressed against the part of the cyst wall already laid down, to form another lamella of layer IV. At least part of the force required to unroll the scrolls is

provided by the muscular flowing motion of the surface of the metacercaria.

The function of the rods has long been a matter of conjecture. Leuckart (1882) believed that they were myoblastic and gave rise to muscle fibres. Rothschild (1936) considered that in the unnamed cercaria she studied, the rods originally played some part in cyst formation but this function had been lost. She did not realise, however, that she had studied only the early stages of formation of the outer cyst wall.

Most of the theories of the function of the rods have been concerned with their role in cyst formation. Sonsino (1884) believed that the cyst wall of an amphistome cercaria which he studied was formed from large numbers of rods laid transversely, side by side. A similar theory was proposed by Barlow (1925) who also reported that the rods were arranged in an orderly fashion by the movements of the metacercaria. Wunder (1932) observed that the rods in Cercaria tuberculata, C. coronata, C. limbifera and C. monostomi, broke down after secretion into a granular and semi-liquid material which formed the cyst wall. A somewhat similar process was described by Singh and Lewert (1959) as occurring during encystment of the cercaria of Notocotylus urbanensis. It seems likely that in these cercariae, the way in which the rods

form the cyst wall will prove to be similar to the unique process described here for F. hepatica.

One of the major unsolved problems of this investigation is concerned with the movement of the secretory cells. With the possible exception of the peripheral mucopolysaccharide cells, it appears necessary to postulate that the gland cells move to the surface to discharge their contents. This would seem to be the only way in which the secretion of those cells originally located almost centrally in the cercaria can pass to the exterior, unless the cells remain in position and extend long pseudopodia to the surface, which seems less likely.

The rod-containing cells certainly have the ability to move, as evidenced by their migration into the space 'vacated' by the tanned protein cells when the embryonic epithelium is charged.

Cell movement is a well known phenomenon in the Turbellaria (Hyman, 1951, p. 78). Pedersen (1958) has described how the totipotent neoblasts migrate to fulfil their reparative function. Skaer (1961) has also described the movement of rhabdite forming cells across the basement membrane to take up an epithelial role.

The procedure envisaged then for the tanned protein and the mucoprotein cells, involves the deeper cells moving to

the surface and discharging their contents. The fate of the remnants of these cells after discharge, is uncertain. It is impossible to decide on the data available, whether the remnants of the cells remain in position close to the surface, or whether they retire to the interior of the body. The first suggestion seems unlikely since there would be an aggregation of nuclei close to the surface which would tend to impede the movement of other cells. The second theory seems equally unlikely if only because the cells after secretion are so badly damaged.

A further question then arises - are the cells regenerated or are they resorbed? At this stage, these questions cannot be answered on the available data, particularly as there are two conflicting considerations to be taken into account. It could be argued that in a small animal, in which such a high proportion of the cells are involved in cyst formation, regeneration of the cells is dictated by future structural needs. This implies that de-differentiation also takes place and it seems unlikely that this is ever possible. Secondly, a large amount of energy would be consumed in this process. Considering the physiological economy of the metacercaria, it would seem more likely that resorption would occur.

The rod-producing cells are not lost but they instead assume a new function, that of providing a tegument for the juvenile fluke. It appears that this tegument consists of a special sort of syncytium in which the nuclei are held below the muscle cells in individual invaginations of the plasma membrane, but most of the cytoplasm forms a layer above the muscles.

The fine structure of the tegument of the adult Fasciola has been recently studied by Threadgold (1963) who reported essentially the same sort of spatial separation of the cellular components. According to Hyman (1951, p. 223), the homology and origin of the cuticle of trematodes have been long disputed. Five theories have been advanced but none is adequate in the light of the knowledge provided by these studies. It seems likely that movement of the keratin cells to the surface allows secretion of the granules which contribute to the inner cyst wall and in addition, by the same process, forms the tegument of the juvenile fluke which is further developed during maturation.

The behaviour pattern of the cercaria from the time it leaves the redia until encystment begins is so complex that in our present state of knowledge it defies analysis.

Perhaps the simplest view is that the behaviour pattern is the result of response to a series of stimuli. Thus the

stimulus to leave the redia may be the charging of the embryonic epithelium.

It is a little more difficult to speculate about the stimulus which causes the cercaria to leave the snail, particularly since there is no information available on the length of time which cercariae spend in the snail tissues before emerging. It is possible that the tanning process, which occurs after transfer of the granules to the embryonic epithelium, may play some part in stimulating the cercaria to leave the snail, perhaps through increasing pressure. It may also be a factor which affects the duration of swimming time prior to encystment.

It is obvious that this area of parasite physiology has great potential interest and is as yet, completely unexplored.

CHAPTER 5

THE EXCYSTMENT OF THE METACERCARIA OF FASCIOLA HEPATICA

INTRODUCTION

The commonest method of infection of the definitive host in the Digenea is by ingestion of a metacercarial stage. The majority of metacercariae live within more or less complex cyst walls and one of the first stages in the process of infection must be the escape of the juvenile fluke (as it can then be called) from the enclosing cyst walls.

Excystment can be considered as predominantly a passive or an active process. Passive excystment implies digestion of the cyst wall by the host enzymes and release of the contained metacercaria, whereas active excystment implies that the metacercaria responds to stimuli from appropriate factors in the gut of the host and actively breaks out of the cyst wall.

Excystment can also be considered from a more general viewpoint as a process connected with resumption of development. Removal of the cyst wall need not necessarily effect

any physiological changes connected with development, whereas this is implicit in the concept of active excystment.

Almost without exception, attempts to excyst metacercariae have assumed that this is a passive process and no cognisance has been taken of the pattern of development. Many of the experiments have been subsidiary to general life cycle studies and have not involved the use of adequate controls. In some cases, the methods and results of such experiments have been reported in so little detail as to make a critical assessment difficult.

Very few in vivo experiments on excystment have been reported. Faust and Khaw (1927) found that excystment of Clonorchis sinensis took place in the duodenum, usually between 14 and 22 hr. after infection. Krull and Price (1932) made a significant observation after infecting frogs with cysts of Diplodiscus temperatus. They found six empty cysts in the rectum, each with a slit-like opening and they concluded that this was evidence of active excystment.

Some of the earliest work in vitro was done using Paragonimus westermani and this has recently been reviewed by Yokogawa et al (1960) and by Dawes and Hughes (1964). The best results were obtained by Oshima et al (1958) and Oshima and Kihata (1958) who found that rapid excystment did not depend on proteolytic enzymes but instead on a stimulus from

low osmotic pressure, high temperature and the presence of bile. Yogore et al (1959) showed however, that excystment would take place in isotonic salt solutions by successive exposure to solutions of pH 3 and pH 9, but the rate of excystment was much slower than reported by Oshima and Kihata (1958). In this species, emergence takes place through a circular hole at the edge of the cyst (Yogore et al, 1959). Ameel (1934) studied excystment in a related species of Paragonimus (kellicotti?) and had initial success with artificial gastric and intestinal juices, but when this failed excystment could be induced in water at 38°C.

The other species which has been studied by several independent workers is C. sinensis. Faust and Khaw (1927) treated cysts with fresh canine gastric juice followed by fresh intestinal juice and observed active movements of the metacercariae, but did not report whether excystment occurred or not. Hsu and Wang (1938) found that successive treatment in artificial gastric juice and trypsin medium resulted in 100% excystment of juvenile C. sinensis. Wykoff and Lepes (1957) reported that excystment occurred in artificial intestinal juice.

Studies on Posthodiplostomum minimum produced conflicting results. Ferguson (1940) found that these metacercariae would excyst in pepsin solution. Hoffman (1958) attempted to

repeat these experiments but found that the best excystment he could obtain was 20% in 8 hr. and this occurred when trypsin digestion followed pepsin pretreatment.

The excystment of the diplostomatid Bolbophorus confusus was also investigated using enzymes (Paperna and Lengy, 1963). Excystment occurred in a trypsin solution, and these workers observed that the metacercaria became active and finally managed to "bore its way out of the dissolving cyst wall".

The excystment of microphallid metacercariae has been studied and it would seem that the stimuli required are very simple. Thus, Hunter and Chait (1952) reported that Gynaecotyla adunca would excyst (70-90% in 1 hr.) in sea water after treatment in an acidified pepsin solution. Ching (1962a, b) found that the metacercariae of Levinsiniella charadriiformis and Maritrema laricola excysted readily in dilute sea water.

Isolated observations on some other species have also been recorded. Krull (1933) observed that digestion of the cyst wall of the metacercaria of Haematoloechus complexus occurred in artificial gastric juice. The excystment of Clinostomum sp. metacercariae was studied by Hemenway (1948) using artificial digestive juices. An average of 31% excysted after treatment with an acidified pepsin solution followed by a neutral trypsin medium. Digestion of the cyst

wall was noted, but Hemenway believed that excystment was active and ascribed this to the muscular movements of the young fluke. When Lengy (1960) exposed cysts of Paramphistomum microbothrium to extracts of the stomach and small intestine of mice, only 58% excysted within 24 hr.

Observations on excystment of the metacercaria of F. hepatica in vivo have been made by a number of investigators. Sinitsin (1914) found that excystment occurred within 2-3 hr. after infection. In 1938 Schumacher conducted a series of experiments with guinea pigs from which he was able to show that the outer cyst wall was removed in the stomach and excystment took place in the small intestine 30 min. to 2 hr. after infection per os.

Dawes (1961) witnessed the excystment of metacercariae removed from mice. He observed that a metacercaria was very active within the cyst and in the latter stages, the oral sucker became attached to the cyst wall and at this point, a hole eventually appeared through which the juvenile escaped. In 1963, Dawes reported observations on sectioned material from the mouse intestine. He believed that the ventral sucker was used to disrupt the cyst wall mechanically.

Some experiments have been carried out on the excystment of Fasciola metacercariae in unusual locations within the host and these have been reviewed by Dawes and Hughes (1964).

Some juveniles excysted when cysts were placed in the peritoneal cavity of mice (Hughes, 1959, 1963; Dawes, 1961). Dawes and Hughes (1964) concluded that "digestive enzymes are not essential for either activation of young encysted flukes or excystment".

It was shown also that metacercariae will excyst in 'foreign' hosts (Dawes, 1961). After administering 50 cysts to each of two chickens, a total of eight flukes were recovered from the abdominal cavity, three days later.

The first experiments on the excystment in vitro of the metacercariae of F. hepatica were recorded by Wright (1927), who used artificial gastric and duodenal fluids. No detailed results were given but according to Wright, they suggested that digestion of the cyst wall takes place in the duodenum and not in the stomach.

Susuki (1931) found that excystment would occur after treatment in an acidified pepsin solution to which a 0.2% bicarbonate solution containing 1% pancreatin and 5-7% ox bile was later added. He demonstrated the viability of the juveniles by infecting goats, rabbits and guinea pigs intraperitoneally.

In 1934, Vogel reported that after treatment in fresh dog intestinal juice at 37°C, 25% excystment occurred within 1-1/4 hr. and 65% after 2-1/4 hr. He injected the juvenile

flukes obtained into the abdominal cavity of a rat, which showed some evidence of infection when killed 41 days later.

Hughes (1959) found that excystment would occur in rabbit intestinal juice, dilute rabbit bile or trypsin solution. After 17 hr. the percentage excystment in each solution was respectively, 84%, 4%, and 14%.

In an attempt to devise a rapid method for testing the viability of Fasciola metacercariae, Wikerhauser (1960) treated cysts with a 0.5% acidified pepsin solution for 2-3 hr. and then added 1% sodium bicarbonate and 0.4% trypsin. The results were not given in any detail but Wikerhauser claimed that 80% of the metacercariae excysted, mostly between 2-3 hr. after addition of the trypsin, but some excysted after 15 min. If bile was excluded from the media, the percentage excystment was very low.

In 1963, Hughes performed some other experiments which were not published although they were referred to in some detail in the review of Dawes and Hughes (1964). The media used were essentially similar to those of Wikerhauser (1960). Cysts were pretreated for 1 hr. in a 1% acidified pepsin solution and then transferred to an artificial intestinal juice composed of trypsin, pancreatin, sodium taurocholate and cholesterol in 1% sodium bicarbonate. After $3\frac{1}{2}$ hr., 50%

of the metacercariae had excysted, after $4\frac{1}{2}$ hr., 55% and after 5 hr., 60%.

One of the problems in defining the factors responsible for excystment is that F. hepatica has been recorded from a wide range of hosts (Appx 1). Although animals such as the sheep, ox and goat are most often found infected, the records show that animals in the orders Perissodactyla, Lagomorpha, Rodentia, Primates and Marsupialia, can also act as hosts. With the exception of two records from the Carnivora and Cetacea which appear doubtful, the animals which can act as hosts are either herbivores or omnivores.

MATERIALS AND METHODS

In this section a general outline of the materials and methods will be given. In cases where special methods were used, the details are discussed separately with the results of each experiment.

Cysts

In the preliminary stages of this investigation cysts obtained from natural and experimental infections of snails were used. All the definitive work was carried out on cysts obtained from experimental infections of laboratory-reared snails; in every case the outer cyst wall was removed before the experiment.

In each experiment, observations were made on at least three replicates, each of ten metacercariae, except where otherwise noted.

Salines

The most commonly used media were based on either Hank's saline or Earle's saline. The Hank's saline was made up without sodium bicarbonate and autoclaved. This will be referred to as Hank's stock saline. When required, sufficient sodium bicarbonate solution, previously sterilized by Seitz filtration, was added to make a 15 mM bicarbonate solution.

The formula for Earle's saline was modified so that the concentration of sodium bicarbonate was 39 mM and the concentration of sodium chloride was adjusted to maintain isotonicity. Seitz filtration was used to sterilize the saline which will be referred to as medium EF.

Normally, 2 ml of medium was used in a Leighton tube (14 ml capacity) fitted with a rubber or silicone rubber stopper.

Bile

Whole sterile sheep bile was used in these experiments. The outer surface of the gall bladder was painted with an alcoholic iodine solution, the bile removed with a sterile

syringe and transferred to sterile bottles. Under these conditions, bile could be stored for several months.

The concentration of bile used was 10% (v/v) which was regarded by Smyth and Haslewood (1963) as a reasonable estimate of the level in the intestine.

In experiments designed to investigate the action of bile, it was found necessary to exclude surface active agents entirely by washing all cleaned glassware, stoppers, etc. through alcohol and ether baths, finally drying at 100°C for at least 30 min.

Reducing Agents

The reductants used were sodium dithionite and cysteine in concentrations of 0.02 M and 0.04 M. The required amount was added in the solid form to each tube.

Gas Mixtures

Gas mixtures were made up commercially as required*. The gas cylinders were fitted with flow meters graduated in litres/min. and the outlet for the gas was through a disposable glass pipette. To equilibrate the medium with the gas phase, the aperture of the glass pipette was held about 2 cm above the surface of the fluid and each tube was gassed

*

The Commonwealth Industrial Gases Limited.

initially for 1 min., allowed to stand, then gassed again for 30 sec. The flow rate of the gas was 4 l/min. Tests of the media showed that the pH agreed with the calculated values and it did not change in 2 hr. This indicated that the medium was equilibrated with the gas phase.

Observations

A cabinet essentially similar to that described by Dougherty et al (1959), thermostatically controlled at 39°C ($\pm 0.8^\circ\text{C}$), was fitted with an inverted microscope so that the eye pieces protruded, permitting observations to be made without any substantial heat loss.

Using Leighton tubes in conjunction with the inverted microscope facilitated observation at high magnifications of the process of excystment.

A 16 mm cine film of the excystment process was taken on a time lapse system (1 exposure every 30 sec.).

pH Determinations

The pH values of media were determined by measuring the pH of at least three replicate tubes not containing metacercariae. Measurements were made with a glass syringe-type electrode.

Procedure

Commonly two media were used and for convenience these will be referred to as the activation medium and the emergence medium.

The activation medium consisted of Hank's saline containing 10% sheep bile, which was then gassed to equilibrium with an appropriate gas mixture in the manner described.

The emergence medium consisted of the modified Earle's saline (EF) gassed with 5% CO₂/N₂.

The required number of metacercariae were placed in the pregassed, warmed activation medium, and the reducing agent added. The tubes were then placed in the warm cabinet and this time was taken as the start of the experiment. After activation for the required time, usually 1 hr., the cysts were removed from the activation medium, washed with distilled water or saline and then transferred to a second tube containing the warmed emergence medium. The cysts were then replaced in the cabinet and observations continued, usually at 15 min. intervals for 1 hr.

Histological Studies

Some observations on sectioned material were made with the light and the electron microscopes. The techniques used have been described in Chapters 2 and 3.

RESULTS

1. Preliminary Experiments(a) in vivo

Some in vivo experiments using rats and mice were carried out simultaneously with in vitro experiments in an attempt to gain some information on the process of excystment in the host.

The more complex experiments involved injection of cysts into the stomach of anaesthetized animals, their recovery after a set time, and injection into the small intestine of other animals for a further period, followed by their recovery and examination. In some cases the cysts were reintroduced into other animals and their recovery and examination repeated. Excystment was poor however, and the only useful observation made was that the outer layer of the inner cyst (layer III), was rapidly digested in the small intestine.

(b) in vitro

Preliminary experiments were concerned with the effects of enzyme solutions on excystment. The enzymes used were pepsin, trypsin, pancreatin and lipase, singly and in different combinations. The results were extremely variable, the first metacercariae usually excysting after at least 3 hr. and the percentage excystment after 24 hr. was often less

than 50%. The details of two typical experiments are given.

Experiment 1:

Fifty metacercariae of F. hepatica were divided into five groups each of ten metacercariae, numbered (a) to (e). Groups (a) to (d) were treated for 1 hr. at 38°C in a 0.25% pepsin (2 x cryst.) solution acidified to pH 2, and then washed in saline. Each group of metacercariae was then transferred to one of the following media and incubated at 39°C:

- Group (a) 0.3% pancreatin (5 x N.F.) in Hank's saline containing 10% sheep bile
- (b) 10% sheep bile in Hank's saline
- (c) 0.3% pancreatin (5 x N.F.) in Hank's saline
- (d) 0.25% pepsin (2 x cryst.) solution acidified to pH 2
- (e) 0.3% pancreatin (5 x N.F.) and 10% sheep bile in Hank's saline. (The treatment of this group differed from that of group (a) in that there was no pretreatment with pepsin.)

The percentage excystment at 24 hr. in the various groups was as follows: (a) 40% (b) 30% (c) zero (d) zero (e) 60%. The first metacercaria in each of the positive groups excysted at 4 hr. (a), 1½ hr. (b) and after 8 hr. (e).

No positive conclusions may be drawn from this experiment but it is obvious that excystment is slow and variable under these conditions.

Experiment 2:

Forty cysts of F. hepatica were divided into four equal groups, each of which were treated for 30 min. at 39°C in a solution of 0.025% pepsin (2 x cryst.) acidified to pH 2. The cysts were then removed from the pepsin solution and washed with saline before transfer to one of the following media:

Group (a) 5 ml Hank's saline with antibiotics added
(100 units/ml of penicillin and 100 mg/ml
of streptomycin)

(b) 5 ml Hank's saline with antibiotics and containing 0.3% trypsin (4 x U.S.P. Pancreatin) and 10% sheep bile

(c) 5 ml Hank's saline with antibiotics and containing 0.5% lipase (448, porcine) and 10% sheep bile

(d) 5 ml of medium (b) containing 0.5% lipase
(448, porcine)

After 5 hr. no metacercariae had excysted in any tube and after 24 hr. the percentage excystment was (a) zero (b) 20% (c) 30% (d) 30%.

It was found that by raising the carbon dioxide content of the gas phase and taking care that the media were equilibrated, the rate of excystment was sometimes improved and the total excystment after 24 hr. was uniformly high. The details of a representative experiment are given below:

Twenty inner cysts of F. hepatica were treated for 1 hr. at 39°C in 0.025% pepsin (2 x cryst.) solution acidified to pH 2, and then transferred after washing in saline to a solution of 0.3% pancreatin (5 x N.F.) and 10% sheep bile in Earle's saline with antibiotics, gassed to equilibrium in the normal manner with 8.8% CO₂/N₂. The first metacercariae excysted at 9 hr. and at 24 hr. the mean percentage excystment in the two tubes was 90%.

Addition of a reducing agent, sodium dithionite or cysteine hydrochloride to this medium resulted in an increase in the rate of excystment. For example, in one representative experiment using 0.02 M sodium dithionite the first metacercariae excysted at 2 hr., at 6 hr. 50% had excysted, and at 24 hr. 100% had excysted. The most striking effect, however, was on the behaviour of the metacercariae. After 15 min. in the medium, all the metacercariae became highly active, and contracted away from the cyst wall. Such a definite behaviour pattern had not been evident in any other media in such a short time. The pH of this medium was between 6 and 7 and shortly

after the metacercariae excysted, they were lysed. It was obvious that some components of the medium had an inimical effect on survival and therefore probably also on excystment. To test this hypothesis, in a further experiment, after activation for 1 hr. in the complete medium, the metacercariae were transferred to medium EF when 100% excysted within one further hour.

From a consideration of these experiments two major points became evident:

(i) The metacercariae would excyst after exposure to (a) a high concentration of carbon dioxide (b) a reductant (c) a temperature about 39°C and (d) bile.

(ii) It appeared that the process of excystment was an active one depending not on passive digestion of the cyst wall by the host enzymes but on stimulation of the metacercaria, resulting in active emergence from the cyst wall.

As a corollary to the second premise, excystment was considered as a two phase process, the initial stimulus provided by the first three factors, i.e. carbon dioxide, reducing conditions and temperature effecting the first phase which will be called activation, the temperature and bile providing the stimulus for the second phase - emergence. Recognition of this concept permitted an investigation into the effects and interactions of the individual stimuli.

2. Factors Affecting Activation

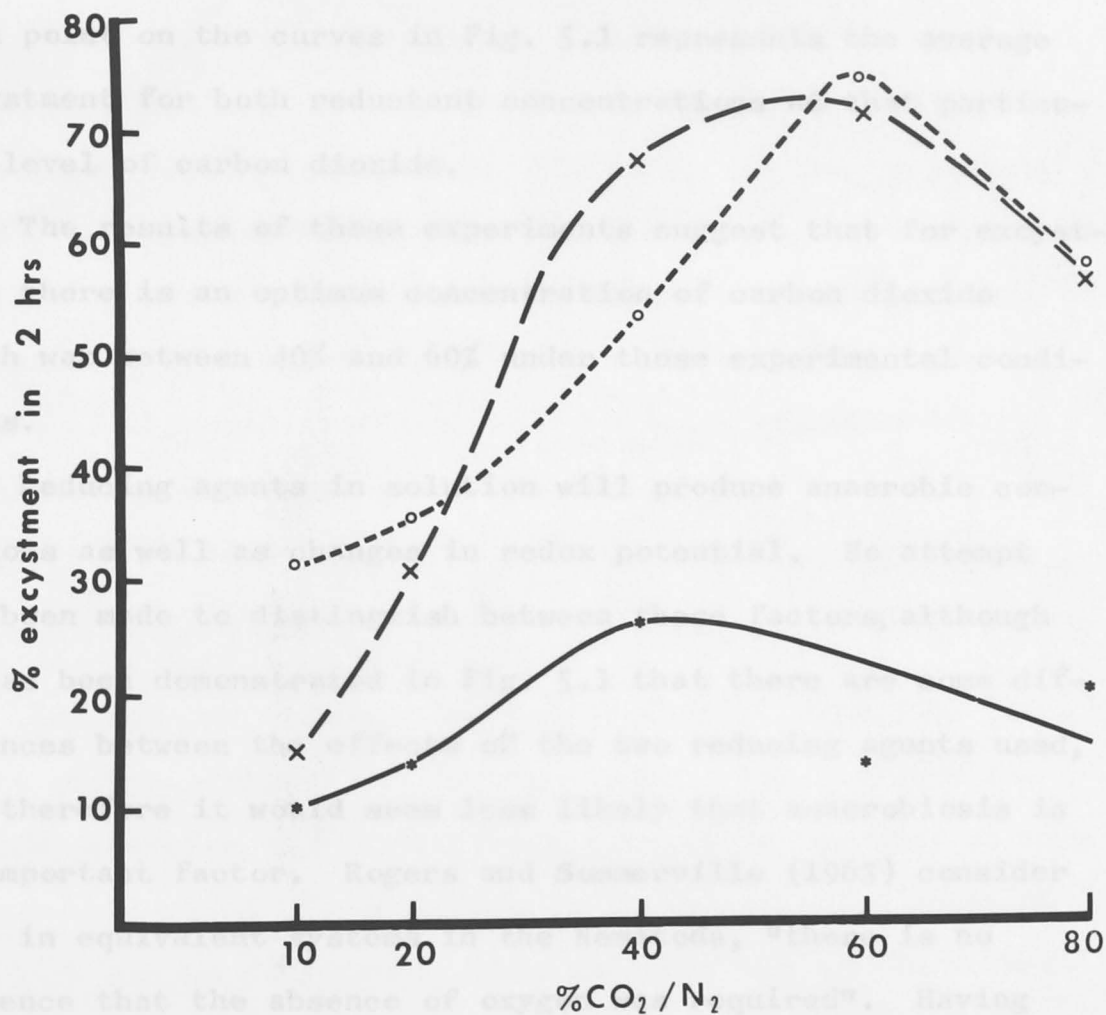
(a) CO₂ concentration and redox potential

Metacercariae of F. hepatica with the outer cyst wall removed were activated in Leighton tubes containing 2 ml of Hank's saline with 10% sheep bile which was gassed to equilibrium in the standard manner with the appropriate concentration of carbon dioxide in nitrogen. In two series of experiments a reductant, cysteine or sodium dithionite in a concentration of 0.02 M or 0.04 M was added. In a third series no reductant was added. After activation for 1 hr., the cysts were transferred to 2 ml of medium EF gassed with 5% CO₂ in nitrogen. Carbon dioxide concentrations of 10%, 20%, 40%, 60% and 80% in nitrogen were used. For each concentration of carbon dioxide and reductant, at least three experiments were performed. The results are shown in Fig. 5.1.

The percentage excystment after 2 hr. of the cysts which were not exposed to reducing conditions increased with increasing carbon dioxide concentration up to 50-60% carbon dioxide, after which it showed some decline. The percentage excystment was never higher than 25%.

When the cysts were exposed to reducing conditions a similar pattern prevailed but the percentage excystment was much higher. Some differences between the results obtained with the different reductants were evident, particularly in

Fig. 5.1: The effects on excystment of increasing carbon dioxide concentrations and of changes in the reducing agent. Unbroken line, no reductant; broken line, 0.02 M and 0.04 M sodium dithionite; dotted line, 0.02 M and 0.04 M cysteine.



combination with the lower concentrations of carbon dioxide, but above about 50% CO₂ the graphs were similar. No differences were observed between the results obtained after exposure to concentrations of reductant of 0.02 M or 0.04 M and each point on the curves in Fig. 5.1 represents the average excystment for both reductant concentrations at that particular level of carbon dioxide.

The results of these experiments suggest that for excystment there is an optimum concentration of carbon dioxide which was between 40% and 60% under these experimental conditions.

Reducing agents in solution will produce anaerobic conditions as well as changes in redox potential. No attempt has been made to distinguish between these factors, although it has been demonstrated in Fig. 5.1 that there are some differences between the effects of the two reducing agents used, and therefore it would seem less likely that anaerobiosis is an important factor. Rogers and Sommerville (1963) consider that in equivalent systems in the Nematoda, "there is no evidence that the absence of oxygen was required". Having noted this, however, in the discussion to follow, for convenience the action of the reductants will be assumed to be limited to the production of reducing conditions.

It is also obvious that a low redox potential is a part of the stimulus. The redox potential of cysteine is probably about -300 mv (Harrison and Quastel, 1928) although other authors prefer a value of about -140 mv (Kendall and Loewen, 1928; Rykjan and Schmidt, 1944). The potential of sodium dithionite has not been measured although it is thought to be a stronger reducing agent than cysteine. It can be assumed that after preparing the tubes using a standard technique, a similar amount of oxygen would be present in each and thus on addition of a standard amount of a reducing agent a similar amount of the oxidised form would be produced. The standard equation for determining redox potential is

$$E = E_o + \frac{RT}{nF} \log \frac{Ox}{Red} .$$

For any one system, the final potential varies as the ratio of the oxidized and reduced forms. For a system involving transfer of two electrons a two-fold increase in this ratio would result in a difference in potential of about 10 mv which can be neglected. This explains the agreement between the results obtained using either 0.02 M or 0.04 M concentrations of the reductant.

It is noteworthy that at carbon dioxide concentrations below the optimum, some differences between the two

reductants were evident but at and above the optimum concentration no differences were apparent. This indicated the possibility that the major part of the stimulus is due to the carbon dioxide, the reductant playing a subsidiary role.

(b) Temperature

The normal activation medium was used with a gas phase of 80% CO₂ and the reductant employed was 0.02 M sodium dithionite. Two temperatures were tested, room temperature (approx. 20°C) for 1 hr. and 26°C for 4 hr. After transfer to the normal emergence medium at 39°C no metacercariae had excysted after one further hour.

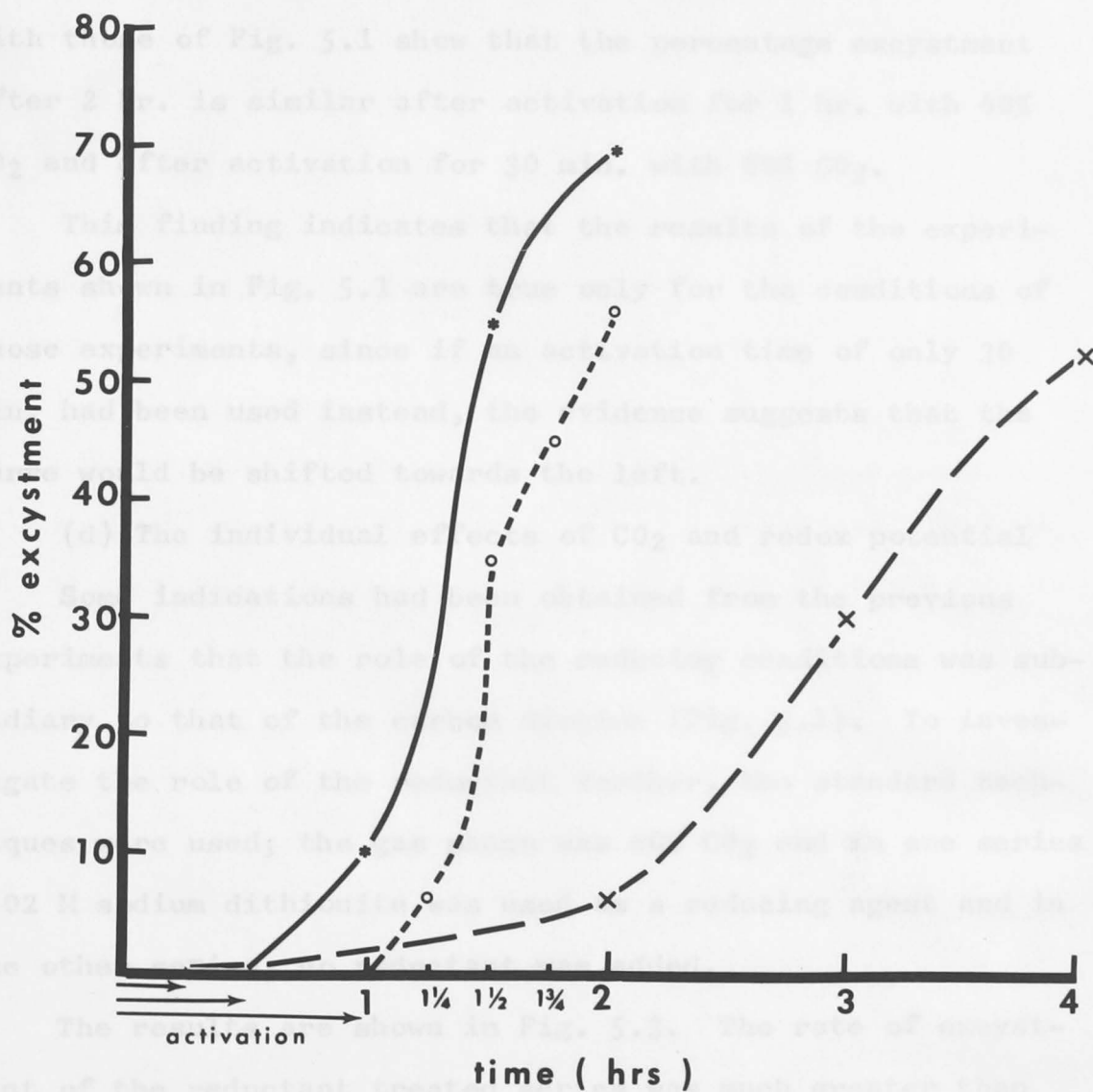
These results show that a temperature about 39°C is an integral part of the activation stimulus and it must act simultaneously with the other components of the stimulus.

(c) Time of activation

In experiments to determine the effect of different activation times on excystment the standard techniques were used; the medium was gassed with 80% CO₂/N₂ and the reductant used was 0.02 M sodium dithionite. The three activation times tested were 15, 30 and 60 min.

The results are shown in Fig. 5.2. Under these experimental conditions, activation for 30 min. provided the optimum stimulus. Activation for 15 min. did not provide a sufficient stimulus and the percentage excystment after 4 hr.

Fig. 5.2: The effect on excystment of varying the time of exposure to the activation medium. The carbon dioxide concentration was 80% and the reductant was 0.02 M sodium dithionite. The three activation times tested were 15, 30 and 60 min., which are indicated by arrows at the bottom of the graph. broken line, 15 min.; full line, 30 min.; dotted line, 60 min.



was only 50%. After activation for 1 hr. the percentage excystment was 55% at 2 hr.

Comparison of the graphs obtained in this experiment with those of Fig. 5.1 show that the percentage excystment after 2 hr. is similar after activation for 1 hr. with 60% CO_2 and after activation for 30 min. with 80% CO_2 .

This finding indicates that the results of the experiments shown in Fig. 5.1 are true only for the conditions of those experiments, since if an activation time of only 30 min. had been used instead, the evidence suggests that the curve would be shifted towards the left.

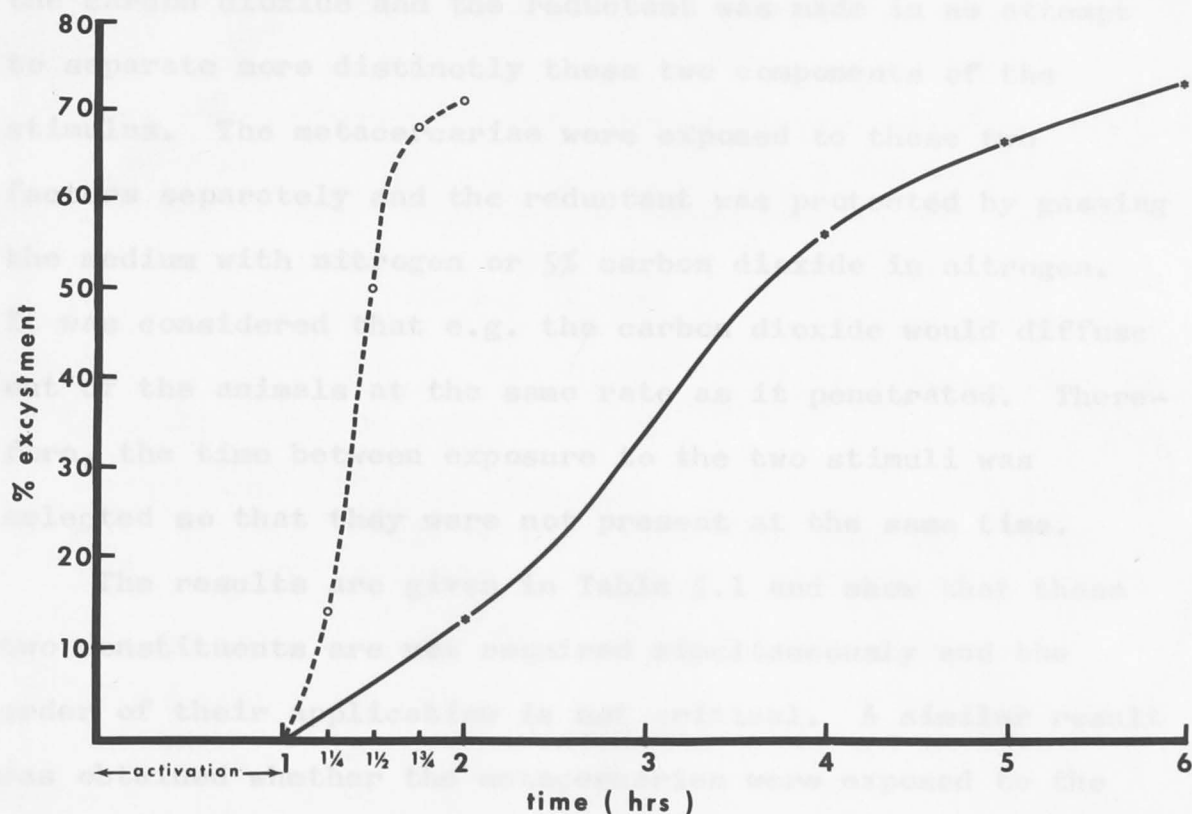
(d) The individual effects of CO_2 and redox potential

Some indications had been obtained from the previous experiments that the role of the reducing conditions was subsidiary to that of the carbon dioxide (Fig. 5.1). To investigate the role of the reductant further, the standard techniques were used; the gas phase was 60% CO_2 and in one series 0.02 M sodium dithionite was used as a reducing agent and in the other series, no reductant was added.

The results are shown in Fig. 5.3. The rate of excystment of the reductant treated series was much greater than that of the other series, although the final percentage excystment was the same in each case.

Fig. 5.3: The effect of reducing conditions on excystment.

Unbroken line, no reductant; broken line, 0.02 M sodium dithionite. The gas phase in each case was 60% CO₂.



It would therefore appear that the carbon dioxide provides the major part of the activation stimulus, the effect of the reductant being to increase the rate of action of the carbon dioxide. A further analysis of the respective roles of the carbon dioxide and the reductant was made in an attempt to separate more distinctly these two components of the stimulus. The metacercariae were exposed to these two factors separately and the reductant was protected by gassing the medium with nitrogen or 5% carbon dioxide in nitrogen. It was considered that e.g. the carbon dioxide would diffuse out of the animals at the same rate as it penetrated. Therefore, the time between exposure to the two stimuli was selected so that they were not present at the same time.

The results are given in Table 5.1 and show that these two constituents are not required simultaneously and the order of their application is not critical. A similar result was obtained whether the metacercariae were exposed to the reducing conditions before or after exposure to the carbon dioxide.

This result permitted a further investigation into the time relationships of the activation process. It was shown that the optimum activation period using 80% CO₂ and 0.02 M sodium dithionite was of the order of 30 min. Experiments were carried out to determine which components of the stimulus

Table 5.1: The effect on excystment of changes in the sequence of application of carbon dioxide and reducing conditions.

Procedure	% excystment at 2 hr.
CO ₂ , 30 min. - reductant, 30 min.	63
CO ₂ and reductant, 30 min. - reductant, 30 min.	80
reductant, 30 min. - CO ₂ , 30 min.	73
no reductant - CO ₂ , 30 min.	33

were required for this time. Metacercariae were exposed to 0.02 M sodium dithionite and 60% CO₂ separately for periods of 30 min. and 5 min. (Table 5.2). The results are also given in this table and it is seen that whereas the required time of exposure to the reducing conditions was of the order of 30 min., a sufficient stimulus due to the carbon dioxide component resulted from exposures for as short a time as 5 min. This exposure time could not be reduced substantially owing to the technical difficulties involved.

These experiments showed therefore, that these two components of the activation stimulus may be separately applied in any order. It would seem likely that the carbon dioxide and reductant affect two systems which although separate have some interaction.

(e) Ionic composition of the activation medium

Metacercariae were activated in either 120 mM or 300 mM sodium bicarbonate solutions which contained 10% bile and 0.02 M sodium dithionite and which were gassed with 40% CO₂. After 1 hr. the metacercariae were transferred to the emergence medium. A third group of metacercariae were activated in distilled water with the same gas phase and the same concentration of reductant. Bile was not added however, until they were transferred to the emergence medium.

Table 5.2: The effect on excystment of varying the individual time of application of carbon dioxide and reducing conditions.

Procedure	% excystment at 2 hr.
CO ₂ , 30 min. - reductant, 30 min.	63
CO ₂ , 30 min. - reductant, 5 min.	30
CO ₂ , 30 min. - no reductant	30
reductant, 30 min. - CO ₂ , 5 min.	70
reductant, 30 min. - no CO ₂	20

The results are given in Table 5.3 and show that

- (i) very large changes in the concentration of bicarbonate ions have no effect;
- (ii) the osmotic pressure of the activation medium can vary within quite wide limits;
- (iii) disregarding the ionization products of the sodium dithionite, specific ions are not necessary.

The activation medium based on distilled water would have contained some bicarbonate ions as a result of the dissociation of carbonic acid (see Appx 2). At this pH (approximately 5.4) the concentration of bicarbonate ions would be very low. In experiments to determine the effect of pepsin treatment (see later) it was shown that metacercariae would become activated in acid solutions (pH 2) under a gas phase containing 80% carbon dioxide. At this pH, no bicarbonate ions would be present and therefore it may be concluded that bicarbonate ions are not necessary for activation.

It is worth noting, however, that the results obtained in these experiments, although satisfactory, were not as high as those obtained using the normal activation medium based on Hank's saline. Perhaps then, although bicarbonate or other ions are not necessary for activation, their presence may enhance the effect of the major stimuli.

Table 5.3: The effect on excystment of changes in ionic composition of the activation medium

[Na HCO ₃]	% Excystment at 2 hr.
-	56
120 mM	56
300 mM	60

(f) Hydrogen ion concentration

It has been shown that metacercariae will become activated in solutions containing high concentrations of carbon dioxide at pH levels between approximately 2 and 8.

Carbon dioxide in aqueous solutions generates hydrogen ions (Appx 2) and so it is difficult to distinguish between carbon dioxide effects and pH effects. However, in one series of experiments, metacercariae which were exposed to reducing conditions and bile in a phosphate buffer pH 6 did not excyst when transferred to the emergence medium. It would therefore appear that the pH of the activating medium is not important.

However, changes in hydrogen ion concentrations affect other factors in the medium. For example, the redox potential varies with pH but the variation is so small it can be neglected in most cases. Hewitt (1950) gives an example of a system in which the Eh:pH curve has "0.03 slope (a very usual value)", and calculates that for a change in pH from 7.1 to 7.2 there is a change in the potential of 3 mv.

Carbon dioxide dissolves in water to form aqueous or dissolved carbon dioxide and some of this combines with water to form carbonic acid which may then ionize with the liberation of bicarbonate and carbonate ions (Appx 2). The effects of bicarbonate and carbonate ions can be studied directly by

controlling the experimental conditions so that these ions are excluded. Separation of the effects of dissolved carbon dioxide and carbonic acid is not possible by direct experimentation. Although the concentration of carbonic acid is very low (about 0.2% of the total carbon dioxide in solution as measured by Taylor (quoted in Jackson, 1964)), this should not be neglected. The sum of the concentrations of dissolved carbon dioxide and carbonic acid can be calculated (Appx 2). In the experiments of Section 2(a) (Fig. 5.1), the pH of the media varied between 5.9 (80% CO₂) and 6.4 (10% CO₂) and therefore the concentration of dissolved carbon dioxide plus carbonic acid varied between 1.43×10^{-2} M and 1.107×10^{-3} M. Further experiments in which the pH is controlled are required to gain a better understanding of the relative effects of pCO₂, dissolved CO₂ and H₂CO₃.

3. Factors Affecting Emergence

(a) Bile

Preliminary experiments had indicated that although sheep bile was an essential component of the complete stimulus it was not a necessary part of the activation stimulus. It seemed likely therefore that it was concerned with emergence and experiments to test this view were set up.

The metacercariae were subjected to the complete activation stimulus, in this case, 80% CO₂ and 0.02 M sodium dithionite for 30 min., after which they were transferred to the emergence medium. After 24 hr. a small amount of sheep bile (approximately 0.2 ml) was added to half the number of tubes, the others remaining as controls.

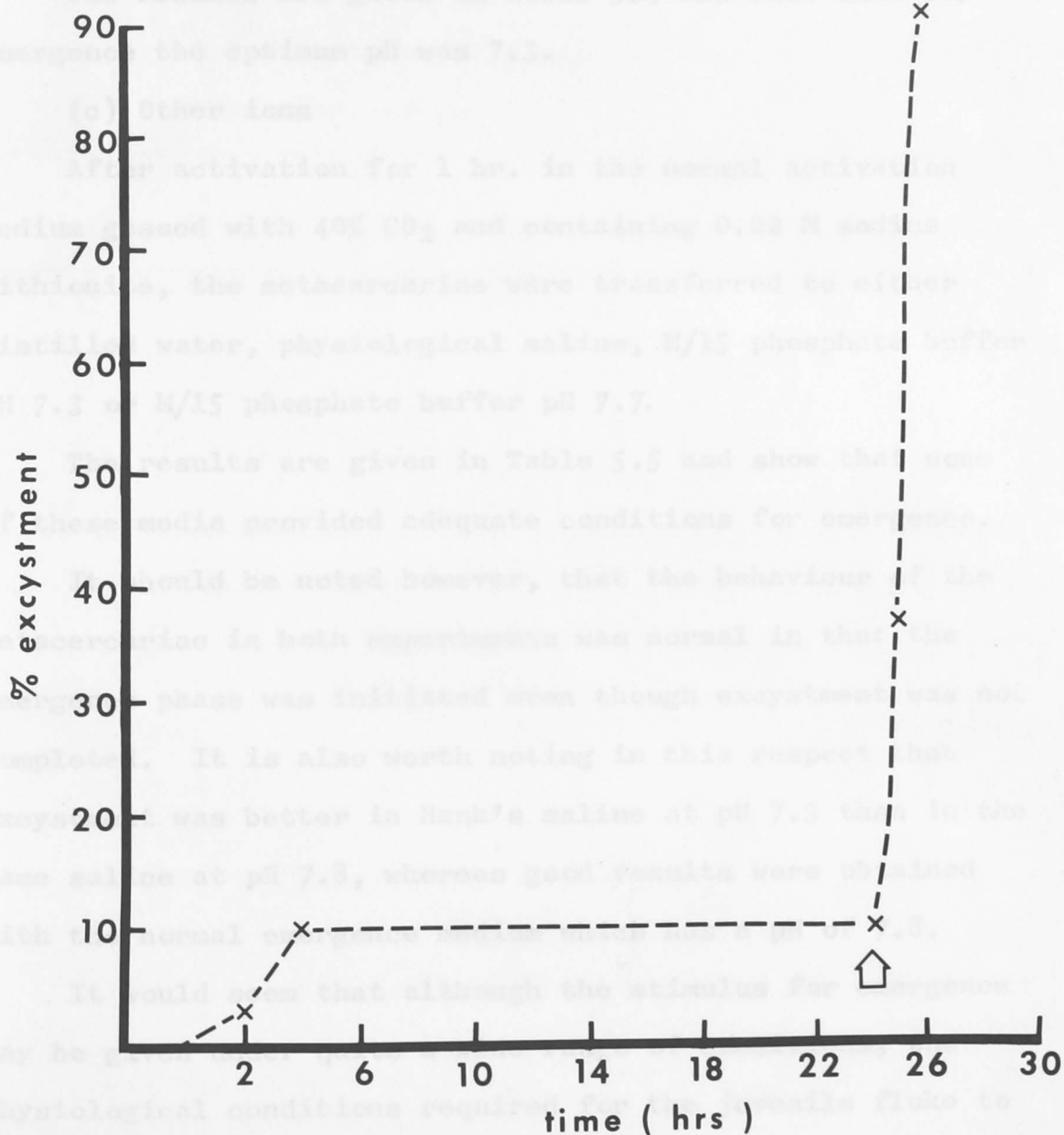
The results are given in Fig. 5.4 and show that final emergence did not take place until after addition of the bile and that then the rate of emergence was similar to that obtained with the standard techniques (cf. Fig. 5.3). During this time no further emergence took place in the control tubes. The viability of the control metacercariae was tested by the addition of bile after 26 hr. which resulted in a mean percentage excystment of 75% at 28 hr.

These experiments demonstrated conclusively that the emergence phase of excystment is triggered by sheep bile and that the metacercariae can remain in an activated state without completing excystment for as long as 24 hr.

(b) pH

The metacercariae were activated in the normal manner using 40% CO₂/N₂ and 0.02 M sodium dithionite for 1 hr. They were then transferred to one of three emergence media based on Hank's saline in which the bicarbonate concentration had

Fig. 5.4: The effect of bile on emergence. Bile was withheld from the activated metacercariae for 24 hr. (arrow).



been altered so that after gassing with 5% CO₂, the pH's of the media were 6.9, 7.3, and 7.8 respectively.

The results are given in Table 5.4 and show that for emergence the optimum pH was 7.3.

(c) Other ions

After activation for 1 hr. in the normal activation medium gassed with 40% CO₂ and containing 0.02 M sodium dithionite, the metacercariae were transferred to either distilled water, physiological saline, M/15 phosphate buffer pH 7.3 or M/15 phosphate buffer pH 7.7.

The results are given in Table 5.5 and show that none of these media provided adequate conditions for emergence.

It should be noted however, that the behaviour of the metacercariae in both experiments was normal in that the emergence phase was initiated even though excystment was not completed. It is also worth noting in this respect that excystment was better in Hank's saline at pH 7.3 than in the same saline at pH 7.8, whereas good results were obtained with the normal emergence medium which has a pH of 7.8.

It would seem that although the stimulus for emergence may be given under quite a wide range of conditions, the physiological conditions required for the juvenile fluke to leave the cyst are fairly precise although nonspecific.

Table 5.4: The effect on excystment of changes in pH of the emergence medium.

pH	% Excystment after 2 hr.
6.9	33
7.3	63
7.8	46

4. The Effect of Enzymes

The effect on excystment of pretreatment with enzyme solutions before normal activation, was tested using a 0.25% solution of trypsin.

Table 5.5: The effect on excystment of changes in ionic composition of the emergence medium

Composition of the emergence medium	% Excystment at 2 hr.
distilled water	35
physiological saline	25
$\frac{M}{15}$ phosphate buffer pH 7.3	25
$\frac{M}{15}$ phosphate buffer pH 7.7	25

4. The Effect of Enzymes

The effect on excystment of pretreatment with enzyme solutions before normal activation, was tested using a 0.25% pepsin (3 x cryst.) solution in Hank's stock saline acidified to pH 2 with 0.1 N HCl and a 0.3% pancreatin (2 x cryst.) solution in Hank's saline (pH 7.4). After successive treatment with each enzyme solution for 30 min., the cysts were washed in distilled water and transferred to the activation medium (80% CO₂, 0.02 M sodium dithionite) for 30 min. and thence to the emergence medium. Some control experiments were also done in which no enzymes were present and in some of these the bile used had been previously heated to 60°C for 24 hr.

The results are given in Table 5.6 and show that the percentage excystment after pretreatment with either pepsin or pancreatin or both is increased relative to the controls. The rate of excystment was also increased, most of the metacercariae emerging within 15 min. after transfer to the emergence medium. An unexplainable result is the increase in excystment which occurs after pretreatment of the cysts with an acid solution. The results with the enzyme solutions would tend to indicate that a nonspecific factor such as, for example, digestion of layer III of the cyst wall, is operating. It has been observed that digestion of this layer

Table 5.6: The effect of enzymes on excystment

Treatment	% Excystment 2 hr. after activation
Pepsin + pancreatin pretreatment	82
pepsin pretreatment	86
pancreatin pretreatment	87
low pH pretreatment	80
5% CO ₂ pretreatment	60
no pretreatment (control) inactivated bile	63

occurs in the duodenum of rats and mice. It is possible also, that acid conditions and proteolytic enzymes may have some slight effect on the ventral plug region. The presence of proteases, lipases or amylases in bile does not influence excystment as the results were similar when normal bile and heat treated bile were used.

Some experiments were also carried out in which the carbon dioxide and reductant were in the acidified pepsin medium. No differences were observed in the rate or in the total excystment.

5. Behaviour

When the metacercariae are activated in the normal activation medium, e.g. gassed with 60% CO₂ and containing 0.02 M sodium dithionite and 10% sheep bile, and then transferred to the normal emergence medium there is a standard pattern of behaviour which appears to be linked with the application of the stimuli.

The pattern of behaviour with the approximate timings is as follows:

- (i) 5-20 min., a period of great activity during which the metacercaria rotates vigorously within the cyst, the ventral sucker sometimes acting as a point of attachment;

Fig. 5.5: The behaviour of the metacercaria during excystment as shown by single frames from a 16 mm cine film.

- (a) activation; the metacercaria rotates vigorously within the cyst wall in the early stages of activation.
- (b) activation; the metacercaria becomes quiescent and imperceptibly contracts away from the cyst wall.
- (c) emergence; after the quiescent phase, the metacercaria becomes active again and the anterior end is thrust against the cyst wall.
- (d) emergence; the metacercaria emerges anterior end foremost through a hole in the cyst wall.

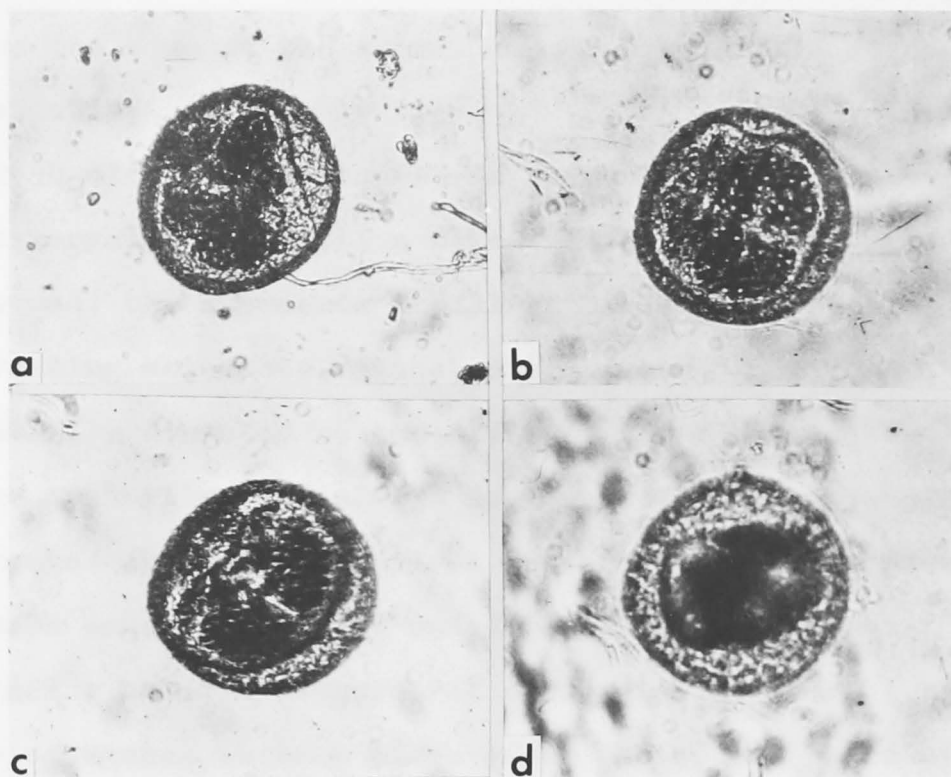
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- (ii) 20-50 min., during this time the movements of the metacercaria gradually cease until there is normally very little movement after about 30 min. After this the metacercaria contracts away from the cyst wall almost imperceptibly, until there is a considerable space - of the order of the thickness of the inner cyst wall - between the edge of the animal and the inner edge of the cyst wall;
- (iii) 50-90 min., the metacercaria suddenly resumes activity but very little rotation takes place if at all, and instead, the movements are almost solely confined to thrusting movements of the anterior end, sometimes initially directed at the sides of the cyst wall but more typically, and always in the latter stages, being directed against the ventral surface. The metacercaria always emerges anterior end foremost through a small circular hole in the ventral surface. The average time for emergence to take place after the second phase of activity has begun is approximately 20 min., although the shortest time observed was less than 5 min. After the metacercaria first starts to emerge through the hole in the cyst wall, it usually takes about 2 to 5 min. to fully emerge. Since the aperture is smaller than the animal, the fluke has to squeeze through the hole and it

sometimes has difficulty pulling the ventral sucker through. In extricating itself, the excretory granules are often forced out of the bladder to remain in the empty cyst. Most metacercariae emerge between 1-1/4 hr. and 1-3/4 hr. after the stimulus is first applied (Fig. 5.3). This is independent of the time of activation and is similar after 30 min. and after 60 min. activation. Treatment with enzymes however, does speed up the process, most metacercariae then emerging between 1 hr. and 1-1/4 hr. after activation. The shortest time taken to complete excystment observed in these studies was 17 min.

The initial phase of movement is in response to the carbon dioxide and temperature as no movement occurs in a bicarbonate solution alone or in combination with enzymes, bile or reductant at 39°C or in a medium with a high pCO₂ at temperatures much below 39°C.

The second phase of movement is triggered by the bile as metacercariae may be held in the fully contracted state for as long as 24 hr. and shortly after the addition of bile the thrusting movements are initiated.

6. Viability of Excysted Juveniles

The average time for which juveniles lived in the emergence medium was about 30 hr. although some remained alive for

as long as 72 hr. As a further test of the viability, 20 freshly excysted juveniles were injected intra-peritoneally into a rabbit and at post-mortem three months later, seven adult flukes were recovered. It can therefore be concluded that the viability of juveniles excysted by this technique is not appreciably impaired.

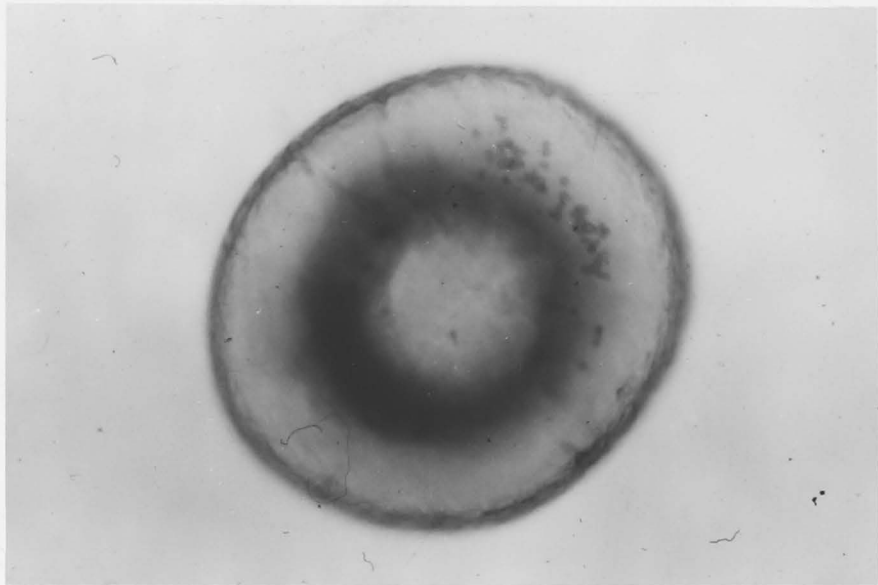
7. Histological Results

When untreated whole cysts were stained with 0.01% toluidin blue for short periods of time, e.g. 2 hr., no stain was taken up. If however, whole empty cysts from which the metacercariae had emerged were treated with the same stain, the area around the emergence hole stained very strongly (Fig. 5.6). This is interpreted as an indication that the chemical structure in this region had been altered resulting in the exposure of end groups which took up the stain.

Observation from the side of activated cysts before emergence, showed that the area corresponding to the emergence hole had a furry, suede-like appearance. Cysts treated at 60°C for 1 hr. and then placed in the normal activation and emergence media do not stain in this manner with toluidin blue, and do not show the pronounced furriness of the ventral surface.

Fig. 5.6: Light micrograph. Whole mount of an empty cyst after the juvenile fluke has emerged. The area around the excystment hole is stained with toluidin blue. Some excretory granules lie within the empty cyst.

Fig. 5.7: Light micrograph. Section through empty cyst after the juvenile fluke has emerged. At the edges of the excystment hole the lamellae of Layer IV are broken. The excystment hole corresponds to the ventral plug region in the intact cyst.



Sections of empty cysts through the emergence aperture (Fig. 5.7) showed that this hole corresponds in position to the ventral plug region in the intact cyst. At the edges of the hole, the cyst wall has a very broken, ragged appearance, and the coarse lamellae which appear to form layer IV, are separated at their ends and thus have a slightly fan-shaped appearance.

Electron micrographs of this region (Figs. 5.8; 5.9) show that layer III appears to have been cut through and the fine lamellae of which layer IV is known to be composed (see Chapter 3) become widely separated and are no longer parallel towards the edges of the hole. In addition, even at the highest magnification, the lamellae were composed of single sheets and thus it would appear that separation of the components of the originally double layered sheet has taken place.

This evidence indicates that digestion of the ventral plug region has probably occurred and has involved the carbohydrate-protein components of layers III and IV and in addition the protein lamellae of layer IV.

Fig. 5.8: Electron micrograph. The appearance of the protein lamellae of the ventral plug region of Layer IV after excystment of the juvenile fluke. The lamellae are widely separated and many free ends are visible. Osmium tetroxide, Reynolds' lead.

Fig. 5.9: Electron micrograph. Higher magnification of the same region as Fig. 5.8. The lamellae are single-layered. Osmium tetroxide, Reynolds' lead.



DISCUSSION

1. The Stimulus in vitro

These experiments have shown that the metacercariae of F. hepatica excyst in response to stimulus from four factors: (a) carbon dioxide, (b) reducing conditions, (c) a temperature about 39°C and (d) sheep bile. Excystment is a two phase process: the first phase, activation, is due to the action of the carbon dioxide, reducing conditions and temperature; the second phase, emergence, is effected by sheep bile. Division of the excystment process into activation and emergence was supported by observations of the behaviour pattern of the metacercariae.

The major part of the activation stimulus is provided by carbon dioxide and temperature. The effect of the reducing conditions is to increase the rate of excystment. Under the conditions of the experiments described, the optimum level of carbon dioxide was approximately 50%. In conditions of low redox potential and low concentration of carbon dioxide, very few flukes became activated and fewer still excysted. It was shown that the metacercariae need only be exposed to a high level of carbon dioxide for 5 min. On the other hand, the required time of exposure to the reducing conditions is of the order of 30 min. The metacercariae can be exposed to the

carbon dioxide and reducing conditions separately and the order of their application can be varied without affecting the result. The available evidence supports the hypothesis that the carbon dioxide and reducing conditions affect two separate but related systems.

Experiments in which the carbon dioxide and reducing conditions were applied separately, also showed that the initial muscular rotatory movements characteristic of the activation phase, are initiated by the carbon dioxide. In reducing conditions alone at 39°C, no movement took place.

The third component of the activation stimulus, temperature, was shown to be essential and in addition is required simultaneously with the other stimuli.

Some other factors, e.g. pH and ionic composition of the medium, did not have any major effect on the action of the stimulus, but in the case of the latter factor at least, some evidence suggested that minor effects might result from variation in these conditions.

The emergence phase depends on the presence of sheep bile and observation of the behaviour of the metacercaria showed that the second cycle of activity which is characterised by thrusting movements of the anterior end, is 'triggered' by bile. The stimulus for emergence could be given under a variety of conditions but the physiological conditions

required to complete excystment were fairly precise although apparently non-specific.

The rate of excystment and the total excystment could be increased by pretreatment with enzymes. This also appeared to be a relatively non-specific effect.

All of these stimuli with the exception of temperature can be considered 'trigger' stimuli in that the response of the organism is manifested after the withdrawal of the stimulus (Bullock, 1957). Fairbairn (1960) has discussed the possible occurrence of these kinds of stimuli in the outside environment and concluded that whereas one or perhaps two of the factors may occur in special situations, the combination of factors is unlikely to be found outside a homoeothermic animal.

It is possible therefore, to define with some precision the stimulus for excystment in vitro and to determine to some degree at least, the way in which the component factors of the stimulus affect the metacercaria. It is more difficult however, to define the stimulus for excystment in vivo although it can be shown that excystment occurs in a similar manner and that the factors responsible for excystment in vitro are also present in the host.

It would be advantageous to compare the percentage excystment in vitro with that in vivo. This has not been

done but it has been shown that juveniles excysted in vitro are able to infect a host.

2. The Stimulus in vivo

One of the difficulties in defining the stimulus in vivo is that F. hepatica in the adult stage parasitizes such a wide range of hosts (see Appx 1). There are very few data available on conditions in the alimentary canal of even the more economically important animals.

Read (1950) has reviewed the literature dealing with the physiology of the vertebrate small intestine, in which he includes data on the composition of the gases of this region. In Table 5.8 the relevant data from this and other sources are summarized.

High concentrations ($> 40\%$) of carbon dioxide are found in the rumen of the sheep, ox and goat, in the stomach of the pig, horse and the one representative marsupial, the quokka. The occurrence of similar high concentrations in the small intestine has only been reported from the horse, the rabbit and man. The values obtained for the caecum and large intestine are often above 40% but the results are difficult to analyse since parts of this system were often compounded.

The redox potentials of different regions of the gut have been determined for only a couple of species. Bergeim et al (1945) have shown that in rats fed on different diets,

the potential of the stomach contents is positive, ranging from + 53 to + 499 mv. The upper portion of the small intestine varied from + 114 to - 194 mv, the lower portion from + 104 to - 235 mv. Hunsgate (1960) indicated that the redox potential of freshly removed rumen contents is - 340 mv while Barnett and Reid (1961) believed this potential to be - 400 mv. It is difficult to generalize from such a paucity of data but it can be said that potentials are negative in the small intestine and large intestine, but in the rumen, the potentials are strongly negative.

According to the in vitro data the optimum activation stimulus is provided by a carbon dioxide concentration of 50% and a redox potential greater than - 300 mv. In the rumen, the average carbon dioxide concentrations vary from 50% in the sheep to 65% in the ox, and the redox potential is of the order of - 350 mv. It is therefore evident that conditions in the rumen would provide a satisfactory stimulus for activation.

From Table 5.8 it is also obvious however, that the carbon dioxide levels in the stomach of the pig, horse, rabbit and marsupial are high enough to promote activation, even though, by analogy with conditions in the rat stomach, the redox potential is probably positive. The negative

Table 5.8: Carbon dioxide concentrations (%) in various parts of the alimentary canal of some hosts of F. hepatica

Animal	Rumen(R)	Stomach(S)	Small Intestine	Reference
sheep	R 50 (28.3-72.8)			Turner & Hodgetts (1955)
	R 70			Washburn & Brody (1937)
	R 72 (68-76)			Tappeiner (1883)
lamb	R 54 (45-72)			"
ox	R 65		18(1st third)	"
goat	R 61 (58-65)		17(1st third)	"
rabbit	S 32		75	"
	S 16		13	Tappeiner (1882)
man	S 4.67-8.68(fasting)			Dunn & Thompson (1923)
	S 12-20		up to 50	Schierbeck (1892)
horse	S 72 (67-75)		42.5(42,43, 1st third)	Tappeiner (1883)
			11(2nd third)	"
			16(last third)	"
pig	S 57 (45-80)		15(10-31, ileum)	Tappeiner (1882)
			27(upper half)	"
			38(lower half)	"
			23	"
quokka*	S 67.5 (60,75)			Moir, Somers & Waring (1956)

* representative of marsupial hosts although not itself recorded as a host.

potential of the small intestine contents and the action of the host enzymes could, however, speed up the process.

The rate of passage of the cysts through the alimentary canal is another factor to be considered in this context. It can be calculated that the rumen clearance time of food particles is about 24 hr. (Hyden, 1955, 1961a, b; McDonald, 1958) and the transit time in the small intestine is 1-2 hr. (Hyden, 1961b). It has been shown in these experiments that the metacercariae can remain in an activated condition for 24 hr. and after receiving the bile stimulus, complete excystment within a further 2 hr.

In non-ruminant hosts, the conditions of carbon dioxide concentration and redox potential in the alimentary canal are such that the time for excystment is likely to be prolonged. Fortunately there are some relevant experimental data available.

Schumacher (1938) fed F. hepatica cysts to guinea pigs and examined them after varying time periods, recording the numbers of unchanged cysts, inner cysts, empty inner cysts and free flukes in different portions of the alimentary canal. Table 5.9 is a rearrangement of this data.

The results suggest that the transit time in the stomach is about 45 min. and in the small intestine is about $1\frac{1}{2}$ hr. They also show that most of the flukes had completed excystment by $1\frac{1}{2}$ hr. By analogy with conditions in the gut of the rabbit,

Table 5.9: The recovery of metacercariae and cysts of F. hepatica after experimental infection of guinea pigs (data from Schumacher, 1938)

			Time(hr.)after infection					
			0.5	0.75	1	1.25	1.5	2
Number and type of cysts	Stomach	unaltered cysts	24	4	-	-	-	-
		inner cysts	29	5	-	-	-	-
		empty inner cysts	5	3	2	-	-	-
		free flukes	5	1	1	-	-	-
and metacer- cariae recovered	Small Intestine	unaltered cysts	6	4	2	1	-	-
		inner cysts	9	36	8	33	1	1
		empty inner cysts	15	61	14	35	22	-
		free flukes	20	26	11	19	12	3
Total all forms*			93	113	26	69	23	4

* Either empty cysts or free flukes taken into consideration, whichever is the greater number.

it is likely that the carbon dioxide concentration is sufficiently high to promote activation and as well, perhaps the low redox potential and the enzymes of the small intestine speed up the process.

It does seem possible however, that in some non-ruminant herbivorous hosts, the rate of passage of cysts through the alimentary canal may be a factor influencing the rate of infection. Larsh (1947), Read (1955) and Read and Voge (1954) have shown that the intestinal emptying time is an important factor affecting the establishment of some tapeworm infections.

Clearly, the length of the small intestine will influence the rate of passage and in carnivores, the small intestine is generally much shorter than in herbivores or omnivores. In addition, it seems possible that the carbon dioxide concentrations in carnivores are much lower. Tappeiner (1882) has recorded a level of 16% in the ileum of the dog, and Robinson et al (1943) have reported concentrations below 10% from several regions of the small intestine of the dog. These two factors acting together, may be important in restricting the host range of this parasite to non-carnivorous animals (see Appx 1).

Schumacher's results (Table 5.9) have some other implications. After 1 hr., all the cysts had left the stomach.

It is interesting to note that some free flukes were found in the stomach region which could indicate that excystment had taken place there, i.e. before receiving any bile stimulus. The maximum number of flukes in the stomach expressed as a percentage of the total number recovered was approximately 3% which is very low and agrees with the in vitro results reported here. Another possible explanation is that of movement of the gut contents during removal of the gut from the body cavity; unless each section was carefully ligated, mixing of the contents of various portions of the gut could easily occur.

The number of unaltered cysts (i.e. cysts with the outer cyst wall still attached) in the stomach fell from about 25% of the total number found at 30 min. to about 5% after a further 15 min. The percentage of unaltered cysts found in the small intestine was also reduced over this time. These data suggest that removal of the outer cyst wall occurs primarily in the stomach. Pepsin does not digest any of the layers of the cyst wall as far as can be observed. Layer I, being composed of tanned protein is impervious to digestion, although Wikerhauser (1960) believed some digestion occurred. Removal of the outer cyst could perhaps be facilitated by digestion of either layer II or III. No digestion of layer III has been observed even after 48 hr. in pepsin solution

and since layer II has roughly the same composition, it seems unlikely that it would be affected either. It is possible that most of the cysts have the outer wall removed mechanically during mastication or during the churning of the stomach or rumen contents, since it is relatively easy to separate the inner cyst from the outer using pressure. If however, the outer cyst is not removed in the stomach, digestion of layers II and III in the small intestine, which has been observed both in the host and in vitro using an artificial intestinal juice, could cause separation of the two cyst walls.

In the in vitro experiments reported here, the outer cyst was removed mechanically from the inner cyst which was then exposed to the stimuli. This would seem in fact to duplicate the situation in the host.

The bile obviously acts to bring the juveniles out in the small intestine. If the stimulus was provided by other factors such as pH, they would excyst in the rumen where the normal pH is similar to that found in the small intestine (Hunsgate, 1960). It would be unlikely that the naked juvenile could survive the acid conditions of the abomasum, but this device ensures that they are able to pass through this inimical environment in an activated but protected condition.

The excystment of metacercariae after injection into the peritoneal cavity as reported by Hughes (1959, 1963) and Dawes (1961), can be explained as a response to continued stimulation from a relatively low concentration of carbon dioxide. Only 10% of the metacercariae excysted and reference to Fig. 5.1 shows that 10% carbon dioxide applied for only 1 hr. would have this effect. Similarly, this level of excystment has been recorded in the absence of bile (Fig. 5.4).

3. The Mechanism of Excystment

It has been shown that the metacercariae excyst through a small circular hole in the ventral side of the cyst. This has been previously reported from in vitro experiments (Hughes, 1959; Wikerhauser, 1960). This method of excystment is also used in the host (Vogel, 1934; Schumacher, 1938; Dawes, 1961).

The evidence from staining reactions, light microscopy and electron microscopy suggests that the hole is formed as a result of digestion of the ventral plug region of the cyst wall.

In considering the source of the digestive substance, it is pertinent to recall firstly, that dead cysts do not show similar changes when subjected to the stimulus, and

secondly, that the process of excystment still takes place when heat treated bile is used. In the face of this evidence it seems likely that the metacercaria secretes a substance which digests the area of the ventral plug.

Dawes (1961) has also maintained that host enzymes are not necessary. Using paraffin sections, he observed the breakdown of part of the cyst wall during excystment, but he attributed this to the physical action of the oral and ventral suckers (Dawes, 1963). These may indeed play some part in the disintegration of the ventral plug region, but it is considered more likely, by analogy with processes in the protozoa and nematodes, that an enzymic secretion of the metacercaria is responsible. The final proof for this will, however, need to be provided by more critical experiments.

4. The Role of Carbon Dioxide and Redox Potential

(a) Other studies on metacercarial excystment

In all other investigations on the excystment of metacercariae, a similar system has not been shown to operate although as has been pointed out already, many of the experiments were poorly designed, particularly with respect to the provision of adequate controls. Thus, in a number of cases, e.g. Susuki (1931), Hemenway (1948) and Wikerhauser (1960), after treatment of the cysts in pepsin solution acidified

with HCl, a trypsin solution containing sufficient sodium bicarbonate to adjust the pH of the medium to about neutrality was added. The reaction of the sodium bicarbonate and HCl would proceed according to the equation



resulting in the liberation of a large volume of carbon dioxide into the medium. The materials and procedure used by Wikerhauser (1960) were stated in sufficient detail to permit calculation of the amount of carbon dioxide released, which would have been approximately 100 ccs at NTP from 200 ccs of medium. Since it has been shown that the time of exposure to high concentrations of carbon dioxide need only be very short, this was probably sufficient to provide part of the activation stimulus and could result in excystment of about 70% after 6 hr. (Fig. 5.3). It would be theoretically possible to achieve a faster rate of excystment if the medium is initially heavily contaminated with bacteria so that the redox potential is quickly lowered to a level where it affects the metacercaria. In a medium containing bacteria, high concentrations of carbon dioxide as well as a low redox potential could also be produced and this could account for the results of Hughes (1959) (on F. hepatica) and Lengy (1960) (on P. microbothrium), who found

that some excystment occurred in intestinal juice after incubation for 17 hr. and overnight respectively.

In further experiments Hughes (1963) found that the percentage excystment after $3\frac{1}{2}$ hr. was 50%, after $4\frac{1}{2}$ hr. 55% and 5 hr. 60%. For similar time intervals from Fig. 5.3, addition of carbon dioxide alone to the medium would have produced 50% at $3\frac{1}{2}$ hr., 60% at $4\frac{1}{2}$ hr. and 65% at 5 hr. The close correspondence of the results would indicate the possibility that a high concentration of carbon dioxide could have been inadvertently applied.

(b) Infection processes of other parasites

The stimulus for activation of the Fasciola metacercaria provided by carbon dioxide, reducing conditions and temperature has also been shown to be at least part of the stimulus for infection in a number of parasites.

Jackson (1962, 1964) found that the first stimulus to excystation of some species of Eimeria is provided by carbon dioxide and temperature but reducing conditions did not have any marked effect. He postulated that the stimulus activated an enzyme system within the sporozoites which acted on the inner wall of the oocyst.

The most intensive work has been done on the hatching of the eggs of some ascarids (Rogers, 1958, 1960; Fairbairn, 1961; Hass and Todd, 1962; Jaskoski and Colucci, 1964) and on

the exsheathment of the infective larvae of some strongyles (Sommerville, 1957; Rogers and Sommerville, 1957, 1960; Rogers, 1960, 1963; Taylor and Whitlock, 1961; Silverman and Podger, 1964). The literature up to 1963 has been reviewed by Rogers and Sommerville (1963). The earlier literature has also been reviewed by Fairbairn (1960) and Rogers (1961).

In these parasites, it has been generally agreed that carbon dioxide provides the major part of the stimulus to hatch or exsheath. Other components of the stimulus were provided by temperature, pH and a low Eh although the last named stimulus was not always essential.

For example, Taylor and Whitlock (1961) and Silverman and Podger (1964) found that high concentrations of carbon dioxide obviated the need for reducing conditions in some cases. Taylor and Whitlock (1961) also showed that various organic acids have a positive effect although somewhat weaker than that produced by carbon dioxide.

All the investigations into hatching or exsheathment of nematode larvae have, according to the authors, indicated the secretion by the larva of an enzyme which digests portion of the egg shell or of the second stage sheath, permitting escape of the larva. This hypothesis was based originally on observations of the effect of exsheathing fluid on intact sheaths and on empty sheaths (see Rogers and Sommerville,

1963; Silverman and Podger, 1964). Sommerville (1957) and Rogers and Sommerville (1960) showed that this fluid was released from the excretory pore or from a region close to this opening. Rogers (1963) has now been able to isolate this enzyme and has shown that one of its components is similar to leucine aminopeptidase. A lipase and a chitinase have also been demonstrated in fluids prepared by hatching large numbers of ascarid eggs (Rogers, 1958, 1963; Jaskoski and Colucci, 1964).

It therefore appears that the high concentrations of carbon dioxide found in the alimentary canal of mammals, act as a signal to the infective stages of many parasites to shed their protective coverings and probably also to resume development. The fact that carbon dioxide is a highly penetrative substance and only occurs in high concentrations in special environments (see Fairbairn, 1960), is probably one of the major reasons why it is active in parasitic groups from different phyla. The response to this factor provides a good example of convergent evolution. It is also interesting that carbon dioxide affects a variety of processes in many organisms (Loomis, 1959; Bartnicki-Garcia et al, 1964).

Some of the possible ways in which carbon dioxide, temperature and redox potential may act on helminth parasites are discussed in Chapter 6.

In every case discussed above, the evidence has suggested that the process of infection is an active one in which enzyme secretions of the parasite digest at least part of the enclosing membranes, thus allowing the parasite to escape to the exterior. Stirewalt (1963) lists some other examples of parasitic helminths which use an enzyme secretion in this way.

By analogy therefore, it would not be unreasonable to suspect that the Fasciola metacercaria also secretes an enzyme which digests portion of the cyst wall. The evidence produced in this study, although circumstantial, would support this view.

5. The Role of Bile

It has been shown that bile is essential for the excystment of Fasciola metacercariae. In the absence of bile, the metacercariae will become activated by the carbon dioxide and temperature stimuli but will not enter the emergence phase.

The significance of bile to parasites has been recently reviewed by Smyth (1962) and Smyth and Haslewood (1963). The latter authors discuss the effect of bile on the excystation of coccidia, the hatching of cestode eggs, the evagination of the cestode scolex and the excystment of metacercariae. In all these processes, bile has been shown to provide at least

part of the complete stimulus for infection. The only literature to be considered here is that which has most relevance, or which was not included in the above articles.

(a) Other studies on metacercarial excystment

Studies on the excystment of some trematode metacercariae have shown that there is an absolute requirement for bile. Thus Kobayashi et al (1959) reported that the metacercariae of Metagonimus yokogawai would not excyst in pepsin or pancreatin solutions alone, but required pig bile.

Wickerhauser (1960) claimed that the metacercariae of F. hepatica would only excyst in the presence of sheep or ox bile. According to Yogore et al (1959) the metacercariae of P. westermani will excyst (75% in 4 hr.) in saline solutions and bile is not required. Oshima et al (1958) produced some similar results but subsequently Oshima and Kihata (1958) were able to show that after addition of 0.5% bile salts to the medium, 100% excystment occurred within 30 min.

The experiments which show that bile is not necessary for the excystment of some metacercariae (e.g. Hunter and Chait, 1952; Ching, 1962a, b) also show that excystment takes place in in vitro conditions similar to those of the stomach, i.e. essentially in media of low pH. An exception to this, is the report of Hsu and Wang (1938) who believed that bile was not required by the metacercariae of C. sinensis although

excystment occurred in a medium containing trypsin near neutral pH. In view of the precautions which it was found necessary to take in the present investigation to exclude surface active agents, a negative result under such conditions should be viewed with suspicion.

(b) Infection processes of other parasites

The excystation of coccidia is increased in media containing bile or bile salts (Jackson, 1962, 1964; Doran and Farr, 1962; Farr and Doran, 1962; Landers and Colley, 1964). In the case of Eimeria arloingi which infects sheep, Jackson (1962, 1964) has shown that excystation is a two-stage process, and the second stage is dependent on bile.

The hatching of cestode eggs is also favourably affected by the presence of bile. Silverman (1954) considered that bile activates the hexacanth embryo to rupture the oncospherical membrane.

According to Read (1955) and Campbell (1963) evagination of the scoleces of some tapeworms occurs rapidly in the presence of bile.

In general, the stimulating effects of bile on nematodes would seem to be limited. Rogers (1958, 1960) believed that sodium taurocholate had a slight positive effect on the hatching of A. lumbricoides eggs, but Fairbairn (1961) showed that this was only evident in older eggs. Jaskoski and Colucci

(1964) also investigated the effect of bile but the conditions of their experiment bear little relationship to conditions in vivo.

Thus, it has been demonstrated quite clearly that bile is an important factor influencing the process of infection in many different parasite groups. The theories which have been advanced to account for the action of bile can be summarized as follows:

1. Bile acts by increasing the effect of enzymes normally present in the gut either by (i) activating the substrate or (ii) activating the enzymes (Doran and Farr, 1962; Farr and Doran, 1962; Silverman, 1954; Read, 1955).
2. Bile acts through its lytic action by increasing the permeability of enclosing membranes (Silverman, 1954).
3. Bile acts by stimulating muscular movement (Silverman, 1954; Read, 1955; Campbell, 1963).

A fourth theory which does not seem to have been considered previously but which on present evidence is tenable is that -

4. Bile acts by increasing the effect of enzymes secreted by the parasite during the process of infection either by (i) activating the substrate or (ii) activating the enzymes.

These theories are, of course, not mutually exclusive. In fact, it is almost certain that the effects of bile in vivo result from the interaction of a number of factors.

In the case of the excystment of the Fasciola metacercaria, bile is an essential component of the complete stimulus for excystment. It is evident that one of the responses of the animal to the presence of bile is a resumption of muscular activity, which is rapidly followed by emergence. Bile may also act according to theory 4 above, by increasing the effect of enzymes secreted by the parasite. The possible effects of bile and its constituents are further discussed in Chapter 6.

CHAPTER 6

SPECULATIONS ON SOME PHYSIOLOGICAL PROCESSES ASSOCIATED WITH EXCYSTMENT

The experimental evidence presented in Chapter 5 has shown that excystment of the Fasciola metacercaria is an active process which is initiated in response to physico-chemical conditions which occur in the gut of the host. Although the stimuli concerned can be categorized with some degree of precision, no information has been gained on their mode of action.

It has been postulated in Chapter 5 that after activation, the metacercaria secretes an enzyme which digests the ventral plug region of the cyst wall. The evidence is largely circumstantial and less equivocal results are necessary to establish the existence of such an enzyme.

It is possible, however, to comment on the possible source of such a secretion. In Chapter 4, it was noted that there are four types of cells in the cercaria which contribute to the cyst wall. A fifth type of secretory cell forms the caecal epithelium.

The caecae of the cercaria of F. hepatica have been described from whole mounts as two continuous sacs. In some descriptions it has been noted that the caecae appear to consist of a longitudinal series of single cells (e.g. Thomas, 1883). However, Sarwar (1958) has reported that the caecae of the cercaria of F. gigantica appear as "two chains of amoeba-shaped granular masses". No observations on sectioned material have been previously made.

In this study, in paraffin sections stained with Mallory's triple stain, the caecae appeared a bright yellow colour (Figs. 4.2; 4.7).

In transverse sections, it appeared on cursory examination that the lumen of each caecum is continuous. However, in longitudinal sections (Fig. 4.2), the caecae have a chain-like appearance, the links of each chain being formed from groups of two or three cells surrounding a central cavity, the lumen of the caecum. Careful examination of serial transverse sections confirmed that the caecum consists of a longitudinal series of dilations. In some transverse sections, both caecae were not visible (Fig. 4.1). In sections of free-swimming cercariae stained with the Mallory-Heidenhain rapid stain, the cells of the caecal epithelium contain large red granules and the lumen is occluded with a homogeneous substance (Fig. 4.3). The cells of the

caecal epithelium reacted positively with the HgBPB test for proteins.

Using the electron microscope, a number of regularly arranged dense bodies were seen in sections (Fig. 6.1) which at higher magnifications, could be distinguished as extracellular spaces, enclosed by two or sometimes more cells (Fig. 6.2). The contiguous plasma membranes of adjacent cells adhere closely, except in the middle regions where they separate to form the extracellular cavity. Septate desmosomes connect the cell membranes on either side of the cavity and appear as a series of dense bars between the plasma membranes which are slightly thicker and denser than elsewhere. Septate desmosomes have been described from Hydra (Wood, 1959) and it is thought that they function in that animal as intracellular attachments and also as a permeability barrier to prevent diffusion of substances between cells. Their function in Fasciola is probably similar. Characteristically, some elements of the reticulum run parallel to the desmosomal region (Fig. 6.2). In many cases, the cells enclosing the extracellular spaces are themselves totally enclosed by thin, flattened cells (Fig. 6.3).

The cytoplasm has a well developed granular reticulum and some Golgi bodies and in addition contains dense, spherical secretory granules which are contained within individual

membranes. The appearance of the cell is that of a typical protein secretory cell (Fig. 4.10). In some cases, the granules could be seen passing through the plasma membranes of the cell into the extracellular space (Fig. 6.4).

These extracellular bodies with the associated cells were visible in sections of cercariae within the snail, free-swimming cercariae, the encysted metacercaria and the freshly emerged juvenile. The extracellular bodies are identified as the lumina of the caecae by their position and by their similarity to the appearance of the caecae in the adult fluke (Gresson and Threadgold, 1959).

The cells therefore form the caecal epithelium and the granules represent a secretion which is passed into the lumen. Dawes (1962) reported that in the adult Fasciola, the cells of the caecal epithelium secrete a substance into the lumen.

It appears likely that the secretion is enzymic and is stored in the lumina so that large quantities can be immediately available. There was no evidence that the mature cercaria is a feeding stage and within the redia, food material is probably absorbed through the surface. However, it can be supposed that the cercaria or the metacercaria could require enzymes to assist in (a) escape from the redia (b) escape from the snail (c) excystment (d) migration

Fig. 6.1: Electron micrograph. Low magnification survey picture of the caecae and the caecal epithelium. The lumena appear as a succession of separate extracellular cavities (lu) filled with a relatively dense amorphous substance. There are numerous granules (g) in the cells which have a well developed reticulum (r) and some Golgi bodies (gb). pm, plasma membrane. Osmium tetroxide, Millonig's lead.

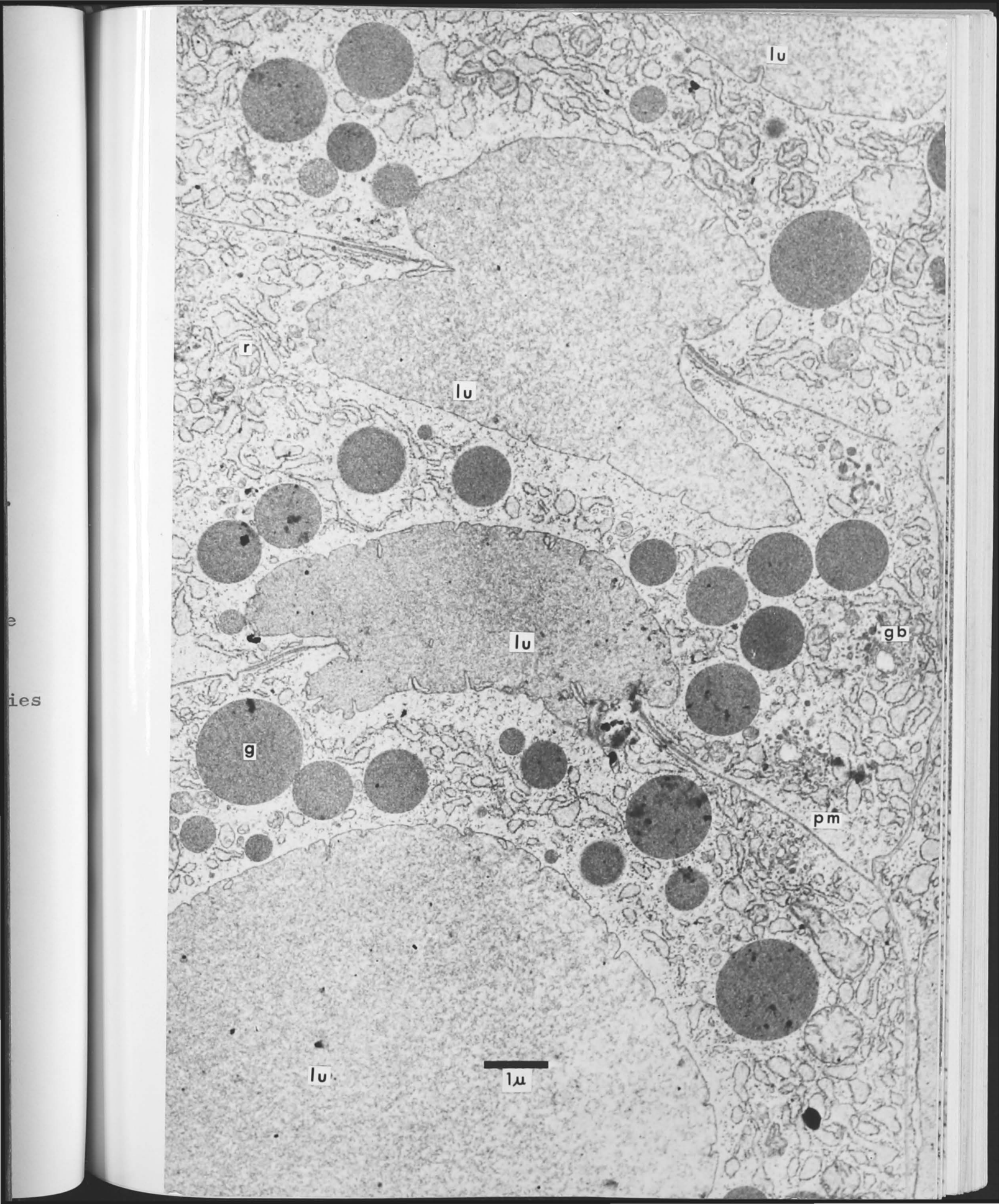


Fig. 6.2: Electron micrograph. The lumen (lu) of the caecum is formed by the divergence of the plasma membranes (pm) of adjacent cells. Either side of the lumen, the plasma membranes are joined together by septate desmosomes (d). Some elements of the reticulum run parallel to this region (arrow). The cells of the caecal epithelium contain mitochondria (m), a well developed particle covered reticulum (r) and some free ribosomes (rb). Conspicuous granules (g) are also present. Osmium tetroxide, Millonig's lead.

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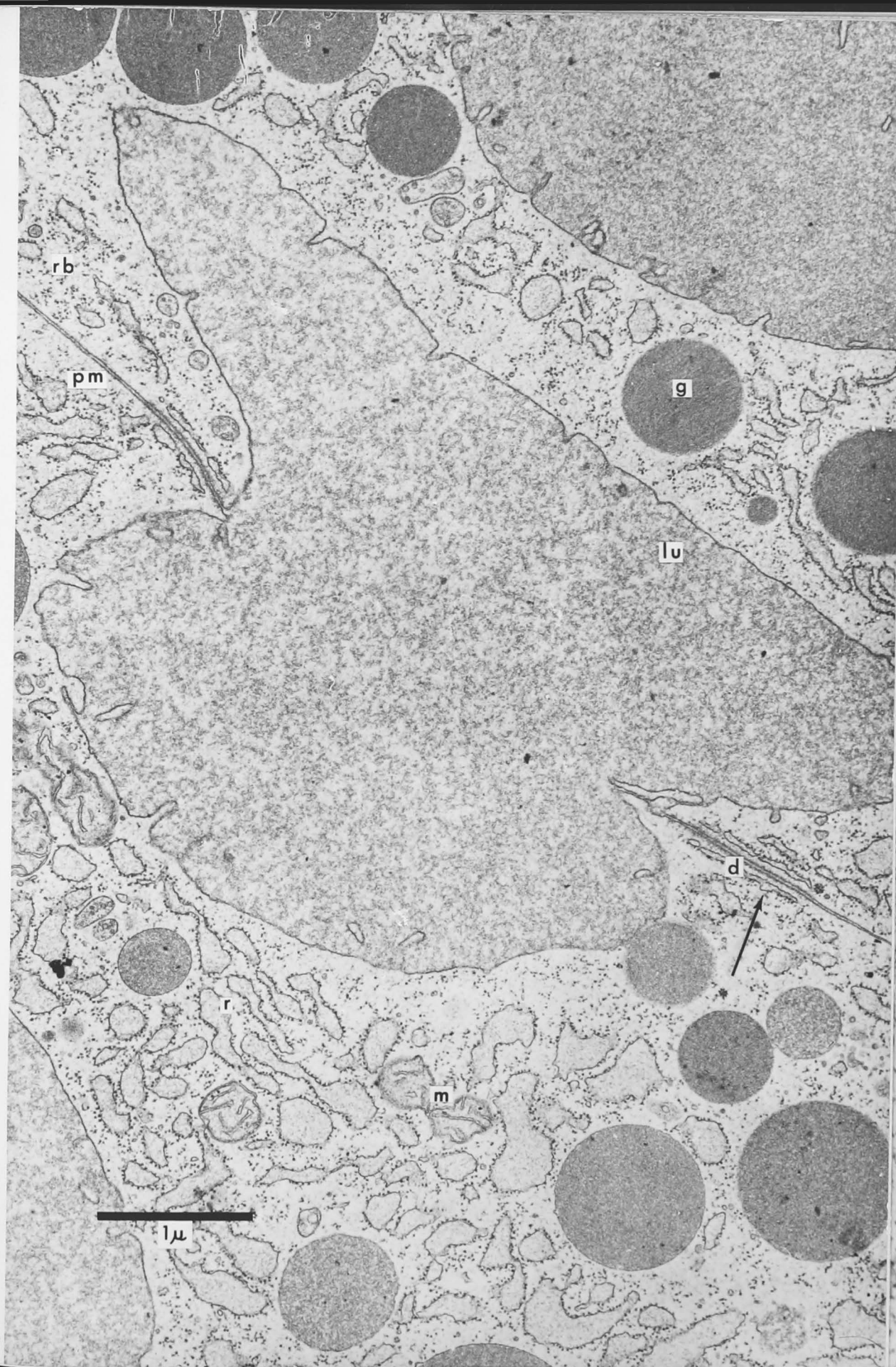
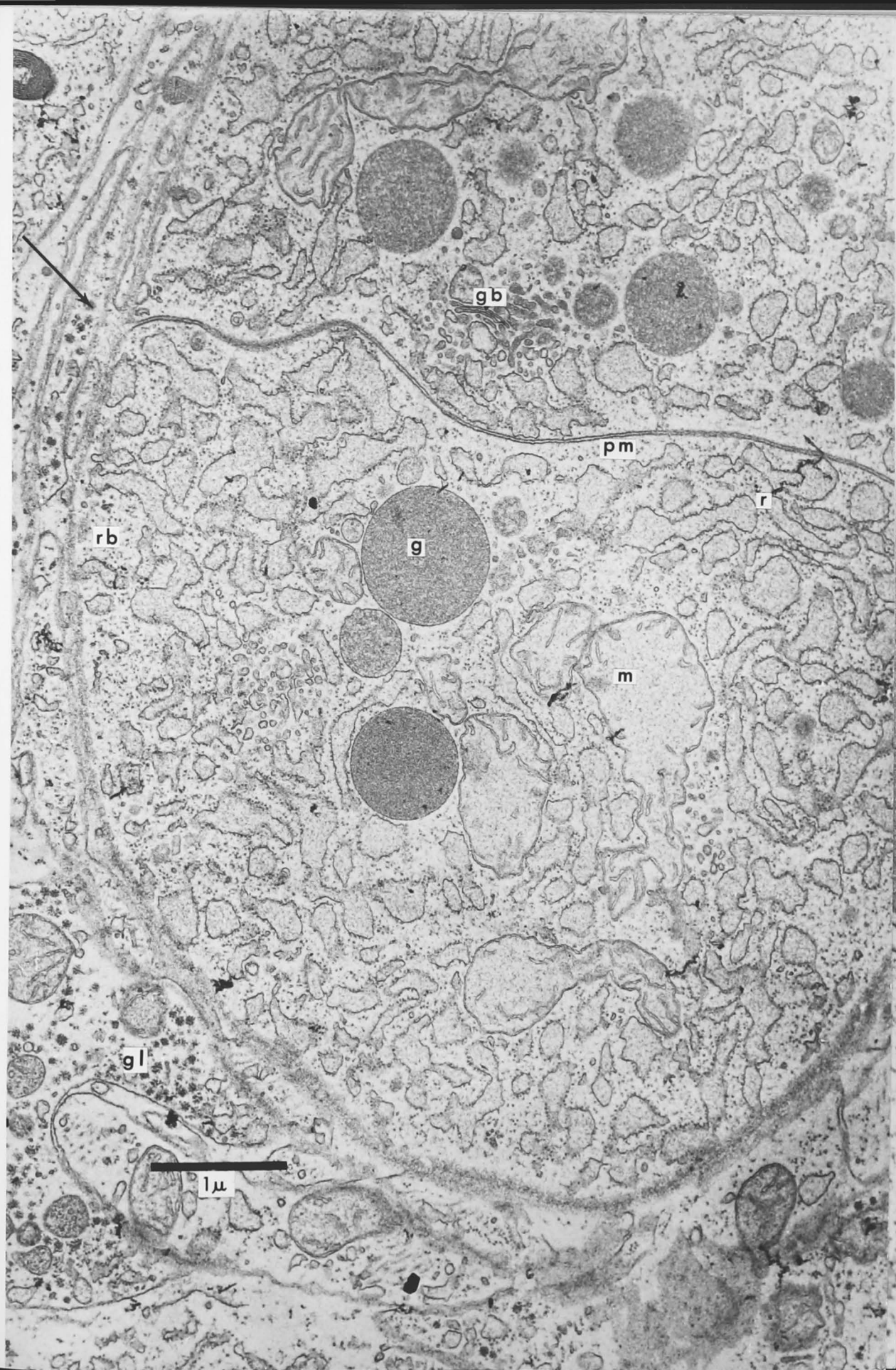


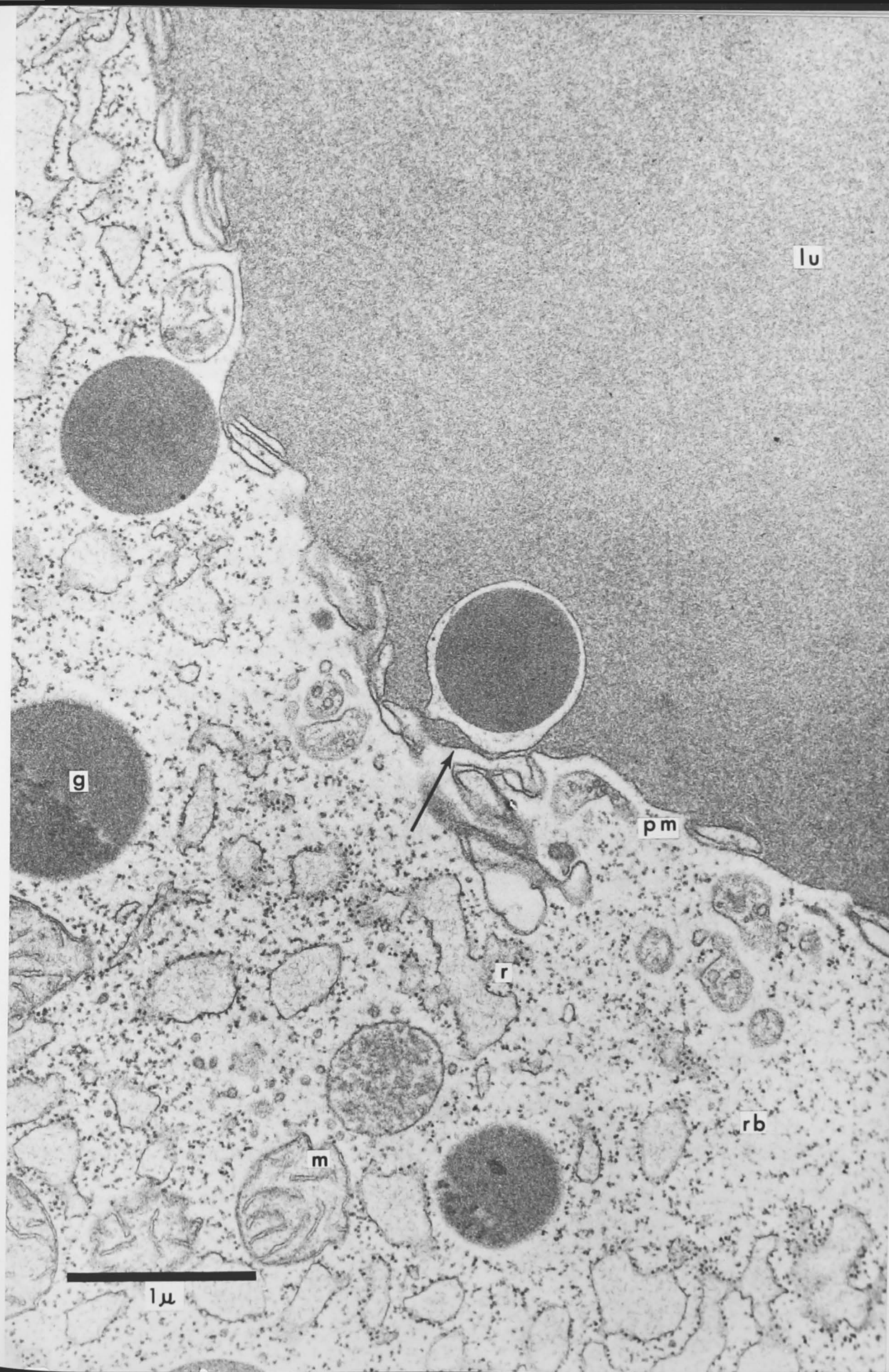
Fig. 6.3: Electron micrograph. The edge of a cell of the caecal epithelium. Another thin flat cell (arrow) is closely attached to the outer surfaces particularly in the region where the plasma membranes (pm) of the epithelial cells adjoin. The flat cells may act to strengthen the contact between the epithelial cells and hold them together. g, granule; m, mitochondrion; rb, free ribosomes; r, reticulum; gl, glycogen particles; gb, Golgi body. Osmium tetroxide, Millonig's lead.



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Fig. 6.4: Electron micrograph. Section through portion of a cell of the caecal epithelium and the lumen (lu) of the caecum. The cell contains a rough surfaced reticulum (r), free ribosomes (rb) and some granules (g). One of the granules (arrow) is passing through the plasma membrane (pm) bounding the lumen. Osmium tetroxide, Millonig's lead.

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through the gut wall. It has been shown that some schistosome cercariae use a secretion in leaving the snail (see Stirewalt, 1963).

According to Hyman (1951, p. 309), extra-corporeal digestion is common in adult flukes and it seems possible that this process may also be used to assist the escape of the cercaria from the redia, the snail and the cyst wall, and as well, the migration of the juvenile fluke.

It has also been emphasized in Chapter 5 that excystment should be considered in a more general light, as a process connected with resumption of development.

It is well known that in some invertebrate phyla, notably the Arthropoda and Annelida, growth and development are under neurosecretory control. Neurosecretion is currently interpreted as the production and release of physiologically active substances by neurons which have the characteristics of gland cells. The evidence available suggests that release of neurosecretory substances involves the same processes, e.g. change in membrane potential, as the generation of action potentials.

In the Nematoda, neurosecretory cells have been described by Gersch and Scheffel (1958) and by Davey (1964). In the Platyhelminthes, neurosecretory cells have been reported by Turner (1946) from Turbellaria and Lender and Klein (1961)

have shown that a secretion from the anterior end may be involved in the regeneration of the eyes of Polycelis nigra. Recently Ude (1962) on the basis of histochemical tests has recorded these cells in Dicrocoelium lanceatum. Dixon and Mercer (1965a) have tentatively identified conspicuous granules occurring in cell bodies and axons in the Fasciola cercaria as neurosecretory. The identification was based on the similarities in fine structure to neurosecretory granules described from other animals.

Correlation with physiological properties or events has been lacking however, except for a significant result reported by Burnett and Diehl (1964a, b). They found that in Hydra, sexual development appears to be inhibited by the secretion of neurohormones which control growth. This result is significant because it demonstrates the presence of a neurosecretory system in an animal which has little or no aggregation of nervous tissue. It is probable that neurosecretory control of growth and development was established very early in the evolution of the metazoa.

The fact that the infective stages of many parasites are also resting stages and that development is restarted in the host has been one of the basic tenets of parasitology for many years. It remained, however, for Rogers (1961) to produce a hypothesis which attempts to explain the biological

processes in the parasite which must be connected with such changes. He has drawn attention to the many similarities between the life cycles of nematodes and arthropods and has speculated that since the control of arthropod life cycles is mediated by a complex system of internal secretions, it seems possible that the control of development in the nematodes is also vested in a neurosecretory system. Rogers has advanced the hypothesis that in the resting infective stages of parasites, "the normal system which controls development is suspended and that a stimulus from the host is required to start it going again".

It seems possible to extend the theory somewhat by considering what constitutes suspension of the system which controls development. Is it merely that the controlling substance has not been secreted, or is there an inhibitory process which blocks secretion? In other words, does the stimulus act directly on the neuron causing it to secrete or does it act by removing an inhibition, thereby allowing the cell to function?

The simplest method by which the stimulus from the host is likely to act is through a receptor which is sensitive to carbon dioxide (Rogers, 1961). Stimulation of the receptor would trigger the release of the hormone and development would resume. These parasites respond to very high levels of

carbon dioxide, and because of its penetrability, it can be expected that the intracellular carbon dioxide concentrations even in the centre of the animal are roughly equivalent to those in the external medium. Chemoreceptors generally are elaborated to detect minute changes in the external environment and therefore there would seem no need to postulate the existence of a receptor. Instead it is possible for the carbon dioxide to affect the neurosecretory cell directly. Chalazonitis (1963) and Chalazonits and Nahas (1965) have shown that action potentials occur in Aplysia neurons exposed to concentrations of CO₂ of 5-50%.

The assumption that the stimulus from the host is directly responsible for restarting development is open to question. In free living animals, it may be assumed that in the primitive condition, the stimulus for release of a growth hormone is due to endogenous stimuli from within the organism itself. For example, in Rhodnius, stretch receptors which are activated by the distension of the abdomen after feeding, control the release of the neurosecretion (see Wigglesworth, 1964). There is no reason to suspect that stimuli from entero-receptors of this nature are not important in parasitic helminths and if their existence is assumed, it then becomes necessary to question why they have no effect. The most likely explanation is that the receptors themselves are

active but their action on the neurosecretory system is inhibited. Van der Kloot (1961) believes that inhibition has been frequently used in the evolution of invertebrate neuro-endocrine systems. He cites examples to show that inhibition may arise through (a) conventional inhibitory neurons, (b) inhibitory neurohormones and (c) the long term inhibition of entire sections of the nervous system. He concludes that in the cases cited, a common principle is that "all involve the long term control of events within the animal" and that "in each case, inhibition is used to insure the animal against a course which would mean complete disaster". He further speculates that "it seems undesirable to rely upon the absence of excitatory stimuli to maintain inactivity. Perhaps excitable tissues are too unstable and liable to spontaneous discharge for this solution to be acceptable. Instead, inhibition is used as insurance against premature activation". Van der Kloot took his examples from the Arthropoda and Mollusca, but in the parasitic helminths the same strictures are apparent and hence it seems reasonable to conclude that similar inhibitory mechanisms may operate.

According to Lorente de No (1948) and Shanes (1948), resting potentials of frog nerves rose in an atmosphere containing carbon dioxide. This may be related to the fall in

intracellular pH (Hadju, 1953) which is probably controlled almost solely by carbon dioxide tension (Caldwell, 1956).

The effect of hyperpolarizations on the parasite nervous system would depend on the inhibitory process in use. For example, the effects of inhibitory neurons can often be removed by hyperpolarizing the membrane. Secondly, if the inhibition relies on long-term depolarization of the neuron involved, as occurs in diapausing Cecropia (see Van der Kloot, 1961), a hyperpolarizing effect could restore activity.

Membrane potentials are also increased when the temperature is raised. This is probably due in part at least, to an increase in the metabolic rate which, in the resting stage, is generally considered to be low. It is also highly likely that the low redox potential and the high carbon dioxide concentrations will also affect metabolic patterns. In other organisms where carbon dioxide is a stimulus to growth or differentiation, it has been suggested that the primary effect is on metabolism, e.g. in the slime mould Blastocladiella (Cantino, 1961) and in Hydra (Lenhoff, 1959; Loomis, 1964).

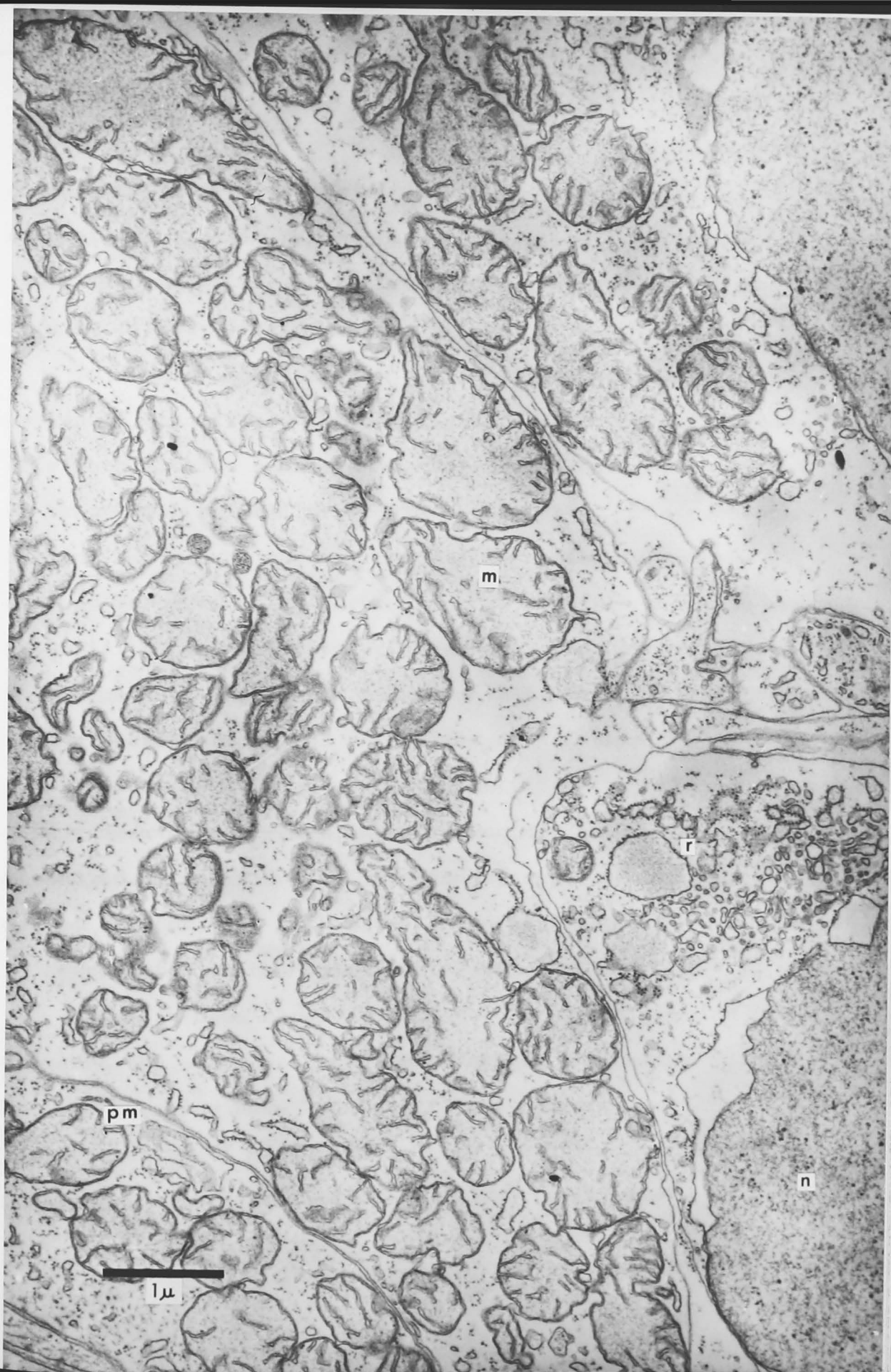
In some adult parasitic helminths living under conditions of low oxygen tension, it has been shown that the electron transport system is reduced, and carbohydrate metabolism does not proceed via the normal Krebs cycle but

via a modified pathway - the Bueding pathway (Bueding et al, 1959; Saz and Vidrine, 1959; Kmetec and Bueding, 1961). Bryant and Smith (1963) suggest that this pathway operates in adult F. hepatica. The suggestion has recently been made that in adult F. hepatica a reduction in the electron transport system may be correlated with a reduction in the number of cristae in the mitochondria (Bjorkman and Thorsell, 1962).

Little is known of metabolic mechanisms in cercariae. From the results of studies on the respiration of various cercariae (see Smyth, 1965), a predominantly aerobic metabolism is indicated. One investigation into the utilization of various substrates by the cercariae of H. quissetensis (Vernberg and Hunter, 1963) suggested the presence of an anaerobic type of metabolism but the results were not conclusive. The fine structure of mitochondria in the cercaria of F. hepatica differs from that in the adult fluke, in that cristae are numerous and well developed (Fig. 6.5). If the number of cristae can be correlated with the possession of the normal 'aerobic' type of electron transport system, the structure of the cercarial mitochondria suggests a normal aerobic metabolism in this larval form.

Therefore if the cercaria has an aerobic metabolism and the adult a modified anaerobic metabolism a 'switch-over' must occur at some stage, and it is most likely that this

Fig. 6.5: Electron micrograph. Mitochondria within a developing cercaria. The cristae are numerous and well developed. m, mitochondrion; r, reticulum; pm, plasma membrane; n, nucleus. Osmium tetroxide, Millonig's lead.



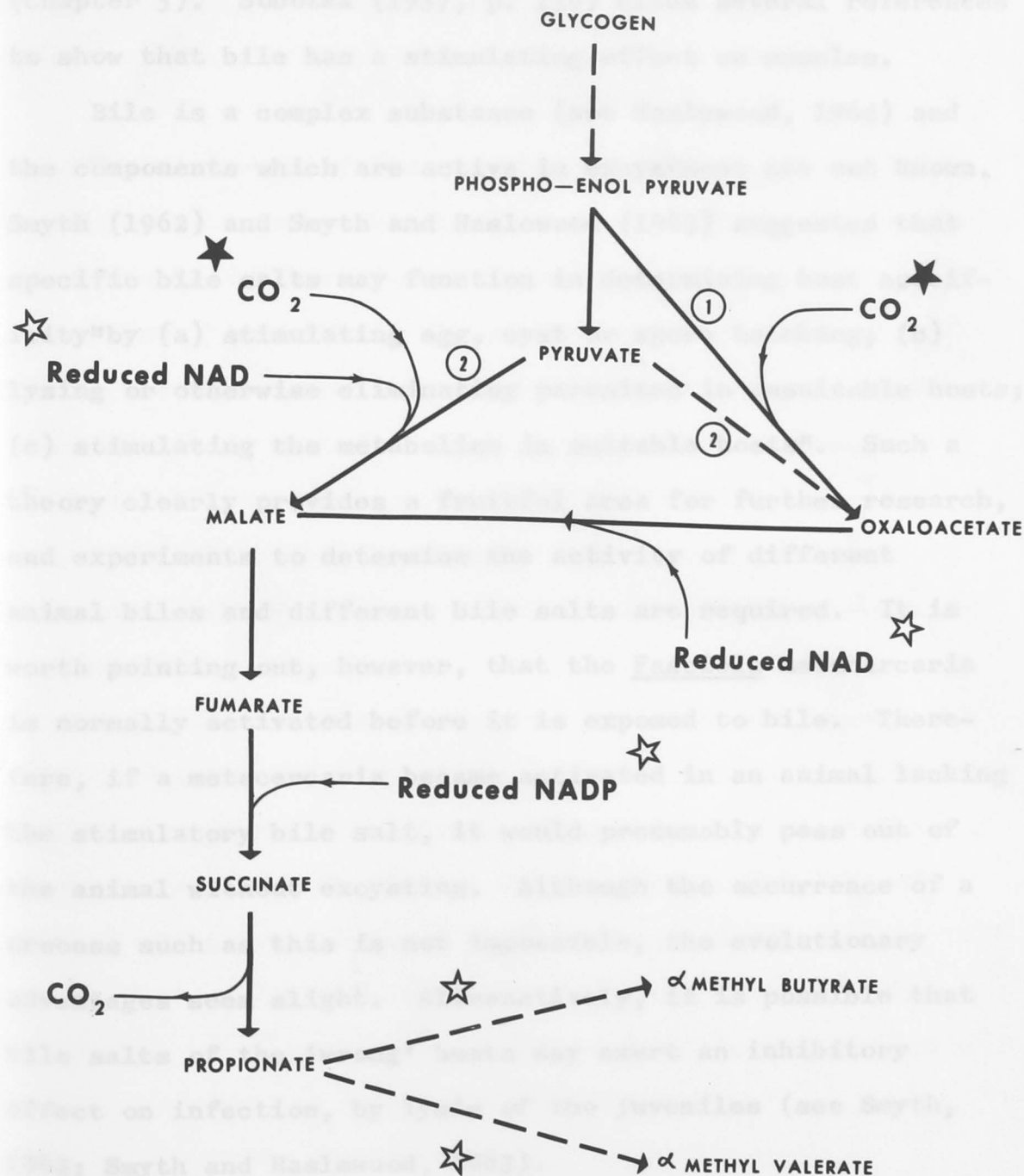
happens when the metacercaria becomes activated. It can be speculated that the imposition of high carbon dioxide concentrations and low redox potentials on the Krebs cycle could lead to metabolic activity being redirected via the Bueding pathway (Fig. 6.6).

In summary therefore, a plausible explanation of the action of the stimuli is that firstly, high concentrations of carbon dioxide remove an inhibition blocking the action of the normal stimuli from entero-receptors. The carbon dioxide may act by raising the resting potential of the neurons involved. The carbon dioxide together with the redox potential and elevated temperature may also have an effect on the metabolic activity of the animal, which in turn would also affect the membrane potentials of neurons.

In Chapter 5, it was pointed out that the effects of carbon dioxide and redox potential on the metacercariae seemed to be individually directed at separate but interconnected systems. To this extent, the hypothesis advanced is in agreement with the experimental data.

Bile has been shown to be a second stimulus which triggers the emergence phase of excystment (Chapter 5). Several theories have been previously advanced to account for the action of bile but the most likely effects on the Fasciola metacercaria are concerned with activation of an

Fig. 6.6: The possible effects of high concentrations of carbon dioxide and low redox potentials on metabolic pathways. The points at which the reducing conditions may exert influence are indicated by stars and the points where carbon dioxide may have an effect are indicated by solid stars.



excystment enzyme and the initiation of muscular activity (Chapter 5). Sobotka (1937, p. 136) cites several references to show that bile has a stimulating effect on muscles.

Bile is a complex substance (see Haslewood, 1964) and the components which are active in excystment are not known. Smyth (1962) and Smyth and Haslewood (1963) suggested that specific bile salts may function in determining host specificity"by (a) stimulating egg, cyst or spore hatching; (b) lysing or otherwise eliminating parasites in unsuitable hosts; (c) stimulating the metabolism in suitable hosts". Such a theory clearly provides a fruitful area for further research, and experiments to determine the activity of different animal biles and different bile salts are required. It is worth pointing out, however, that the Fasciola metacercaria is normally activated before it is exposed to bile. Therefore, if a metacercaria became activated in an animal lacking the stimulatory bile salt, it would presumably pass out of the animal without excysting. Although the occurrence of a process such as this is not impossible, the evolutionary advantages seem slight. Alternatively, it is possible that bile salts of the 'wrong' hosts may exert an inhibitory effect on infection, by lysis of the juveniles (see Smyth, 1962; Smyth and Haslewood, 1963).

CHAPTER 7

SUMMARY AND CONCLUSIONS

The cyst wall of the metacercaria of F. hepatica is a complex structure consisting of four major layers, one of which can be further divided into three sub-layers.

Layer I is a thick, incomplete, external layer which covers the metacercaria dorsally and laterally. It is composed of tanned protein, which is arranged as a meshwork of irregular bodies made up of cigar-shaped particles.

Layer II is a thin, fibrous layer closely adherent to the inner surface of layer I and is composed of mucoprotein and acid mucopolysaccharide.

Layer III is made up of three separate sub-layers, the relative widths and composition of which vary slightly in different regions of the cyst. In general, IIIa consists of mucoprotein, IIIb of acid mucopolysaccharide and IIIc of neutral mucopolysaccharide. In the electron microscope, IIIa can be distinguished because of its denser, more granular appearance but layers IIIb and IIIc cannot be distinguished

between, and appear as a single, less dense, slightly fibrous layer.

Layer IV, in the dorsal and lateral regions, appears to be formed of lamellae, held in a protein-lipid matrix. The lamellae are composed of protein stabilized by disulphide linkages. In the electron microscope, the layer is seen to be formed from numerous, parallel protein sheets, and aggregations of these give the lamellar appearance seen in the light microscope.

The ventral part of layer IV is thickened and gives reactions for neutral mucopolysaccharides. In the electron microscope, some protein lamellae are seen in this region also but the distance between adjacent lamellae is greater than elsewhere in layer IV. It was speculated that more free ends would be found in this region.

In the cercaria, there are four major types of gland cells which can be related histochemically and electron microscopically to specific layers in the cyst wall.

The tanned protein cells occupy most of the ventral half of the cercaria and are filled with large, yellow-brown globules which give positive reactions for phenols and basic protein. These are two of the three components of a tanning system. The fine structure of the granules is that of an aggregation of dense, cigar-shaped particles. The cells

have a well developed reticulum and the granules develop within vesicles.

The mucopolysaccharide cells are relatively few in number and are arranged in rows on the dorsal surface, except for a few around the ventral sucker. The contents are made up of acid and neutral mucopolysaccharide. In the electron microscope, the granules appear to be of several types, although some of them may be transitional stages. The cells have numerous Golgi bodies and a poorly developed reticulum exhibiting some polarization; the granules develop within vesicles.

The mucoprotein cells are situated roughly centrally in the anterior half of the body. They number about 10-12 and the granules formed by them are probably composed of mucoprotein. The granules are not enclosed within membranes and characteristically appear broken. The mature cells contain a poorly developed reticulum and no Golgi bodies were seen. Cells in the early stages of synthesis were not recognized.

The keratin cells occupy most of the dorsal half of the body and contain numerous parallel rod-like granules which are arranged in cubical 'packets'. The rods give reactions for protein, sulphhydryl groups and disulphide bonds. In the electron microscope, the rods appear as tightly rolled-up,

double-layered protein sheets. Each rod is contained within a membrane and in their development, the rolling-up process occurs simultaneously with addition to the sheet. The cells have the normal equipment of proteogenic cells.

Within the redia the cercariae are enclosed by an embryonic epithelium composed of a single layer of large flat cells. Before the cercariae migrate into the snail tissues, the contents of the tanned protein cells and some of the granules of the mucopolysaccharide and mucoprotein cells are secreted into the embryonic epithelium, which can then be considered equivalent to the pre-formed outer cyst wall. The tanned protein granules are secreted through channels between groups of muscle cells. After secretion of this material, the keratin cells move to occupy almost the whole of the body.

After the cercaria leaves the snail, it swims about for a short time before encysting. Encystment can be divided into several phases:

- (i) an exploratory phase during which the cercaria moves about over the substrate;
- (ii) an attachment phase when the cercaria flattens itself against the surface using the ventral sucker as a point of attachment; the ventral parts of the embryonic epithelium appear to become stuck to the substrate, and the tail breaks off;

- (iii) a contraction phase when the metacercaria draws away from the outer cyst wall;
- (iv) an active phase during which the metacercaria rotates actively within the forming cyst wall.

When the embryonic epithelium is cast off, the cigar-shaped particles of the tanned protein granules move relative to each other along 'fracture lines' and then adjacent granules adhere to form the loose meshwork of layer I. The initial rotatory movements of the metacercaria draw the carbohydrate-protein granules into long, poorly formed fibres characteristic of layer II.

The mucopolysaccharide and mucoprotein cells contribute their contents to form layer III. Although the details of the formation of this layer were not fully elucidated, it seems likely that the mucoprotein cells form the outermost layer IIIa, and the mucopolysaccharide cells form the layers IIIb and IIIc. The granules of the mucoprotein cells appear to be secreted to the exterior through channels between the muscle cells, and the mucopolysaccharide cells probably use the same method.

The keratin cells containing their scroll-like particles move towards the surface and extend pseudopodia-like processes between the muscle cells to the exterior, to form a layer overlying the muscle cells. The scrolls contained within

their vesicles move towards the outer surface where the vesicle membrane fuses with the plasma membrane, thus releasing the scroll to the exterior, where it is unrolled to form a sheet. As each sheet is unrolled it is pressed against the preceding sheet to form a dense series of closely packed parallel lamellae. The unrolling of the scroll is assisted by the muscular rotatory movements of the metacercaria.

It is considered that the migration of the keratin cells to the surface to form a layer overlying the muscles, forms at the same time, the tegument of the juvenile fluke.

Excystment did not occur after enzyme treatment and it was found that four stimuli are required:

- (a) high concentrations of carbon dioxide
- (b) low redox potential
- (c) bile
- (d) a temperature about 39°C.

Excystment is a two stage process, activation and emergence. Activation is triggered by carbon dioxide, reducing conditions and temperature. The major stimulus comes from the carbon dioxide and temperature. The effect of the reducing conditions is to increase the rate of action of the other two stimuli. The carbon dioxide stimulus need only be applied for 5 min. but the time of exposure to a low redox

potential has to be of the order of 30 min. Changes in the order of application of the stimuli, carbon dioxide and redox potential have no effect.

The second phase, emergence, is triggered by the presence of bile in the medium. It was shown that metacercariae can be held in an activated condition for as long as 24 hr. and then, after addition of bile, emergence takes place normally.

The metacercariae exhibit a complex behaviour pattern when activated. There is an initial period of rotatory activity but after about 20 min. this gives place to a period of quiescence, during which the metacercariae contract away from the cyst wall at an imperceptible rate. This is followed at about 50 min. after exposure, by a second phase of activity, consisting of antero-posterior thrusting movements which are directed against the ventral region of the cyst wall. Within about 15 min. the juvenile flukes escape through a small circular hole in the ventral surface of the cyst wall. The first phase of activity is in response to stimulus from carbon dioxide, the second phase in response to stimulus from the bile.

The hole through which the juvenile fluke emerges corresponds to the ventral plug region of the cyst wall. Alteration in staining reactions and changes in the fine structure of the protein lamellae indicate that chemical

activity has occurred in this region, and it is postulated that the metacercaria, in response to stimulus, releases an enzymic secretion which digests the ventral plug region of the cyst wall. It was speculated that the cells of the caecal epithelium are the source of the enzyme.

The conditions necessary for excystment in vitro were compared with conditions in the gut of the host. It seems likely that in the primary definitive hosts - the Ruminantia - activation takes place in the rumen and emergence takes place in the duodenum below the opening of the bile duct. In non-ruminant hosts, activation may take place in the stomach or small intestine. The rate of passage of food material along the gut may influence infection rates but it was shown that the normal proteolytic enzymes of the gut will increase the rate of excystment. High concentrations of hydrogen ions similar to those found in the stomach also have a similar effect.

The possible modes of action of carbon dioxide and reducing conditions in activating the metacercaria were discussed in the light of Roger's theory (1961) on neurosecretory control of development. An hypothesis was advanced that these factors may act by removing an inhibition, thereby allowing the normal endogenous stimuli for further development to act.

The manner by which bile affects the emergence phase was discussed. It may activate an enzyme secreted by the parasite and secondarily, may induce muscular movements in the metacercaria.

APPENDIX 1

THE DEFINITIVE HOSTS OF FASCIOLA HEPATICA

A search of Helminthological Abstracts (1932-64) was made to provide a list of the animals recorded as hosts of F. hepatica during that time. The more common hosts, sheep and cattle, were excluded as also was man. The results are summarized in Table 1, where the number of references for each host are given and as well, the most recent reference.

This parasite has also been recorded from kangaroos (Johnston, 1909), wallabies (Johnston, 1911) and monkeys (Bezubik and Furmaga, 1959). According to Price (1932), there are two records of F. hepatica from marine mammals. Stiles and Hassal (1894) recorded this species from Orca gladiator and Stiles (1894) identified a fluke originally found by Leuckart in a "Schwert-fisch" as F. hepatica. Stiles believed the host was probably Orca gladiator. Price considered that some error may have occurred in labelling and in any case, it has been shown that the description of the host was inadequate. Delyamure (1955) has created a new species F. skrjabini for F. hepatica Stiles and Hassal 1894

from Orca orca (= O. gladiator) and Balaenoptera acutorostrata.

In some text books, e.g. Dawes (1946), the cat, dog and elephant are listed as hosts of F. hepatica although references are not cited. It has not been possible to verify these reports in spite of a search of Zoological Records prior to 1932.

It may be concluded then that F. hepatica is able to parasitize a wide range of hosts including representatives of six orders: Artiodactyla, Perissodactyla, Lagomorpha, Rodentia, Primates and Marsupialia. Some species of three other orders, the Cetacea, Proboscidea and Carnivora may also possibly act as hosts.

Table 1: Animals recorded in Helminthological Abstracts
(1932-64) as hosts of F. hepatica

Host	Order	No. of records	Most recent reference
goat	Artiodactyla	3	Balasingham (1962)
camel	"	1	Hoeppli & Wu (1952)
buffalo	"	7	Ezzat <u>et al</u> (1963)
deer	"	2	Schmid (1938)
pig	"	7	Kovacs & Nemeseri (1958)
horse	Perissodactyla	7	Turner (1961)
ass	"	2	Collins (1961)
mule	"	1	Edwards (1943)
rabbit	Lagomorpha	4	Bono & Pellegrini (1959)
hare	"	3	Malanowska (1962)
coypu	Rodentia	4	Holmes (1962)
squirrel	"	1	Savin (1960)
rat	"	2	Macchioni (1962)
guineapig	"	2	Trofimov & Alyabeva (1959)

APPENDIX 2

CARBON DIOXIDE IN AQUEOUS SOLUTIONS

A full treatment of this subject is given by Edsall and Wyman (1958). It is important to these investigations to determine the amounts of carbon dioxide and carbonic acid in the medium.

According to Henry's law,

$$[\text{CO}_2]_{\text{tot}} = Q \text{ pCO}_2 \quad (1)$$

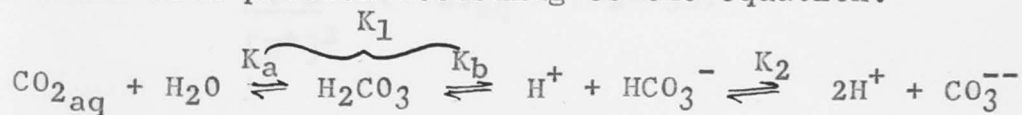
$$\text{where } [\text{CO}_2]_{\text{tot}} = [\text{CO}_2]_{\text{aq}} + [\text{H}_2\text{CO}_3] + [\text{HCO}_3^-] + [\text{CO}_3^{--}] \quad (2)$$

expressed in moles;

Q is Henry's law coefficient and

pCO_2 is the partial pressure of carbon dioxide in the gas phase in atmospheres.

The hydration of carbon dioxide and the dehydration of carbonic acid proceed according to the equation:



where K is the ionization constant.

$$K_1 = \frac{[H^+] [HCO_3^-]}{[CO_2]_{aq} + [H_2CO_3]}$$

$$\therefore [HCO_3^-] = \frac{K_1 ([CO_2]_{aq} + [H_2CO_3])}{[H^+]} \quad (3)$$

$$K_2 = \frac{[H^+] [CO_3^{--}]}{[HCO_3^-]}$$

$$\therefore [CO_3^{--}] = \frac{K_2 [HCO_3^-]}{[H^+]}$$

substituting for $[HCO_3^-]$

$$[CO_3^{--}] = \frac{K_1 K_2}{[H^+]} \frac{([CO_2]_{aq} + [H_2CO_3])}{[H^+]} \quad (4)$$

Substituting values for $[HCO_3^-]$ and $[CO_3^{--}]$ in equation (2)

$$[CO_2]_{tot} = [CO_2]_{aq} + [H_2CO_3] + \frac{K_1}{[H^+]} ([CO_2]_{aq} + [H_2CO_3]) +$$

$$\frac{K_1 K_2}{[H^+]^2} ([CO_2]_{aq} + [H_2CO_3])$$

$$= ([CO_2]_{aq} + [H_2CO_3]) \left(1 + \frac{K_1}{[H^+]} \left(1 + \frac{K_2}{[H^+]} \right) \right)$$

and substituting for $[CO_2]_{tot}$ from equation (1)

$$Q \text{ pCO}_2 = ([\text{CO}_2]_{\text{aq}} + [\text{H}_2\text{CO}_3]) \left(1 + \frac{K_1}{[\text{H}^+]} \left(1 + \frac{K_2}{[\text{H}^+]} \right) \right)$$

However at pH values less than 8 the ionization of HCO_3^- is suppressed.

Therefore for $\text{pH} < 8$ equation (5) becomes

$$Q \text{ pCO}_2 = ([\text{CO}_2]_{\text{aq}} + [\text{H}_2\text{CO}_3]) \left(1 + \frac{K_1}{[\text{H}^+]} \right)$$

similarly at pH values less than 5, ionization of H_2CO_3 is suppressed.

Therefore for $\text{pH} < 5$ equation (5) becomes

$$Q \text{ pCO}_2 = ([\text{CO}_2]_{\text{aq}} + [\text{H}_2\text{CO}_3])$$

This treatment holds for carbon dioxide in distilled water. Addition of salts affects the solubility of carbon dioxide and the presence of bicarbonate ions in the medium will depress the ionization of carbonic acid.

Example

To calculate the concentrations of aqueous carbon dioxide and undissociated carbonic acid in a medium in equilibrium with a gas phase of 10% carbon dioxide and with a pH of 6.4

$$(\text{CO}_{2\text{aq}} + \text{H}_2\text{CO}_3) = \frac{Q \text{ p CO}_2}{1 + \frac{K_1}{[\text{H}^+]}}$$

From table of Q values (Edsall and Wyman (1958) p. 55)

$$Q = 0.025$$

$$\text{pCO}_2 = 0.1 \text{ atmospheres}$$

$$\text{pH} - \text{pK}_1 = -\log [\text{H}^+] + \log K_1 = \log \frac{K_1}{[\text{H}^+]}$$

pK_1 for H_2CO_3 is 6.302 at 38° (Edsall and Wyman (1958) p. 558)

$$6.4 - 6.3 = 0.1$$

$$\text{antilog } 0.1 = 1.259 = \frac{K_1}{[\text{H}^+]}$$

$$([\text{CO}_2]_{\text{aq}} + [\text{H}_2\text{CO}_3]) = \frac{0.025 \cdot 0.1}{2.259}$$

$$= 1.107 \times 10^{-3}\text{M}$$

This calculation neglects the effects of other ions in solution although, with the exception of bicarbonate ions, the effect is likely to be small.

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